Spirulina platensis AND *Chlorella vulgaris* IN PRACTICAL DIETS OF JUVENILE AFRICAN CATFISH (*Clarias gariepinus*)

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

INSTITUTE OF BIOLOGICAL SCIENCES
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Spirulina platensis AND *Chlorella vulgaris* IN PRACTICAL DIETS OF JUVENILE AFRICAN CATFISH (C*larias gariepinus)*

ABSTRACT

Aquaculture production in natural or controlled marine or freshwater environment is an important industry for food security. Extensive production of African catfish is highly dependent on fishmeal (FM) as the most preferred protein source owing to its well-balanced nutrient properties. However, increasing prices of FM and the need for more secured alternative nutritional sources have aroused significant interest in the use of plants sources, especially the algae. This study evaluated the potential of *Spirulina platensis* and *Chlorella vulgaris* in the diets of African catfish (*Clarias gariepinus*) in relation to nutrient and amino acids digestibility, growth performance, immune-stimulatory and antioxidant properties. Four different feeding trials were conducted with *Spirulina* (SP) and *Chlorella* (CL) replacing up to 75% fishmeal (FM) protein contribution in the various feeding trials except for digestibility experiment where the two test diets were formulated using 30% each of the test ingredients (SP and CL) and 70% reference (Ref containing FM and plant feedstuffs) diet on a dry basis. Results obtained from these trials indicated that SP and CL exhibited significantly higher ($P<0.05$) nutrient and amino acids ADCs than FM experimental group. CL75% best-supported growth and Polynomial curve estimation regression analysis revealed the optimum FM replacement levels by SP and CL to be 68.5% and 69.4% respectively. These levels subsequently served as a guide in feed formulation of immunity and oxidative stress enzymes studies. The effect of SP on flesh fat deposition was found to be dependent on the nutritional composition, the lipid source and quantity (plant/ animal oil) as well as the quantity of SP incorporated into the diets. Comparatively SP75% and CL50% had better haematological and biochemical values pre-and post- *Aeromonas hydrophila*, than control. Increased supplementation of both algae significantly reduced LDL (58.65-

58.68mg/dl) and enhanced HDL (69.21 – 70.66mg/dl) an indication of their hypocholesterolemic properties. Post-challenge mortalities were significantly decreased in groups treated with both algae (26.67- 53.33%) as compared to control (80%). Significantly highest intestinal (1.29×10^5 Cfu ml^{-1}), and lowest liver (3.72×10^2 Cfu ml^{-1}) bacteria load was found in the control and CL50% respectively. Also, maximum lysozyme and respiratory burst activity were observed in CL75 (145U ml^{-1}) and CL50 ($1.55A_{540}$) correspondingly. This indicated that the different algae inclusion levels enhanced the immunity and survivability of the African catfish. Replacement of FM by *Spirulina* and *Chlorella* in African catfish diet improved CAT and SOD (especially in CL75% and SP75%) activities of the Africa catfish despite the minor effect of GST enzymes. This suggests that substituting FM with 50 – 75 % of SP or CL, in the diets of *C. gariepinus*, have the potential to improve growth and feed efficiency with no adverse effect on the fish's well-being. This thesis, therefore, stands to argue that introduction of SP and CL are strongly recommended as a partial replacement for the conventional FM in Africa catfish feed. The introduction of both algae would enhance higher output, improve the revenue base of the entrepreneur in the African catfish industry and increase food animal protein for the consumers.

Keywords: *Spirulina-platensis*, *Chlorella-vulgaris,* Fishmeal, Practical-diet *C.-gariepinus*

Spirulina platensis DAN *Chlorella vulgaris* SEBAGAI PERMAKANAN PRAKTIKAL BAGI IKAN KELI AFRICA (*Clarias gariepinus*) JUVENIL

ABSTRAK

Pengeluaran akuakultur secara alami, marina terkawal ataupun sekitaran air tawar adalah industry penting keselamatan makanan. Pengeluaran pesat ikan keli Afrika adalah sangat bergantung kepada makanan ikan sebagai sumber protin utama disebabkan oleh ciri zat nutriennya yang seimbang. Walaubagaimanapun, kenaikan harga makanan ikan dan keperluan sumber nutrisi alternatif yang lebih selamat telah meningkatkan minat yang mendalam atas penggunaan sumber tumbuhan Septembererti alga. Penyelidikan ini telah menilai potensi *Spirulina plantensis* dan *Chlorella vulgaris* sebagai makanan bagi ikan keli Afrika (*Clarias gariepinus)* di dalam aspek penghadaman nutrisi dan asid amino, prestasi pertumbuhan, peransangan imun dan ciri-ciri antioksida. Empat jenis percubaan pemakanan yang berbeza telah dijalankan dengan *Spirulina* (SP) dan *Chlorella* (CL) menggantikan sehingga 75% sumbangan protin bagi makanan ikan di dalam percubaan pemakanan yang pelbagai untuk ujikaji penghadaman dimana dua cubaan pemakanan diformulasikan menggunakan 30% setiap juzuk makanan (SP dan CL) dan 70% rujukan (rujukan mengandungi makanan ikan dan tumbuhan pada pemakanan) pemakanan (7:3) pada asas kering. Hasil ujikaji yang diperolehi daripada percubaan ini menunjukkan bahawa SP dan CL memberi kenaikan beerti ($P<0.05$) pada nutrisi dan asid amino berbanding kumpulan ujikaji pemakanan ikan. CL 75% telah menampung pertumbuhan terbaik dan analisa anggaran lengkuk polinomial menunjukkan tahap penggantian makanan ikan bagi keduanya SP dan CL adalah 68.5% dan 69.4%, masing-masing. Kesan SP ke atas pengumpulan lemak didapati bergantung kepada komposisi nutrisi, sumber lemak dan kuantiti (tumbuhan/minyak haiwan) dan juga kuantiti SP yang dicampurkan bersama pemakanan. Secara perbandingannya. SP75% dan CL50% nilaian hematologi dan biokimia terbaik pada pra dan pasca aeromonas hydophila daripada

kawalan. Kenaikan penambahan kedua-dua alga telah mengurangkan LDL (58.65-58.68 mg/dl) secara siknifikan dan menambahkan HDL (69.21-70.66 mg/dl) sebagai petunjuk kepada ciri-ciri hipokolesterolomiknya (26.67- 53.33%). Kematian pada pasca cabaran adalah berkurang secara siknifikan bagi kumpulan yang dirawat dengan kedua-dua alga. Kepadatan bakteria adalah didapati tinggi di dalam perut kumpulan kawalan (1.29 X 10^5 Cfu/ml) manakala kepadatan yang paling kurang didapati pada hati bagi yang diberi makan dengan CL50% (3.72 X 10^2 Cfu/ml). Maksima aktiviti lisozim dan aktiviti ledakan pernafasan adalah diamati pada CL75 (145U $^{ml-1}$ dan CL50 ($1.55A_{340}$). Ini menunjukkan bahawa penambahan alga yang berbeza menambahkan system imun semulajadi yang mana menyumbang kepada kelangsungan hidup ikan keli Afrika. Penggantian FM kepada *Spirulina* dan *Chlorella* pada pemkanan ikan keli Afrika meningkatkan aktiviti CAT dan SOD (terutamanya pada CL75% dan SP75%) pada ikan keli walaupun kesan hnaya kecil pada aktiviti enzim GST. Ini mencadangkan bahawa SP dan CL, pada tahap penggantian 50-75% FM di dalam pemakanan *C. gariepinus* mempunyai potensi menambah-baikkan pertumbuhan dan efisiensi pemakanan tanpa memberi kesan negatif pada ikan. Tesis ini, oleh itu, berhujah bahawa pengenalan SP dan CL adalah sangat disyorkan sabagai penggantian Septemberara bagi FM bagi ikan keli Afrika. Oleh itu, pengenalan kedua alga akan menambahkan hasilan, menambah-baikkan pendapatan berdasarkan industry ikan keli Afrika dan meningkatkan nutisi protin bahan makanan bagi komsumer.

Kata Kunci: *Spirulina-platensis Chlorella-vulgaris* Tepung-ikan, Diet-praktikal Ikan-keli

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LIST OF SYMBOLS AND ABBREVIATIONS

AAC	Amino acid coefficient
ADC	Apparent digestibility coefficient
A/GR	Albumin/globulin ratio
AL	Alanine Transaminase
ALB	Albumin
ALP	Alkaline phosphatase
AST	Aspirate transaminase
BW	Body weight
BWG	Body weight gain
CAT	Catalase
CF	Crude fibre
CFU	Colony forming units
CL	Crude lipid
CP	Crude protein
Crt	Creatinine
C.V	*Chlorella* vulgaris
DCP	Di calcium phosphate
Df	Dilution factor
EAA	Essential amino acid
EFA	Essential fatty acid
FAO	Food and Agriculture Organization
FM	Fishmeal
FW	Final weight
GE	Gross energy

Hct	Haematocrit
HDL	High-density lipoprotein
Hgb	Haemoglobin
HIS	Hepatic somatic index
HPLC	High performance liquid chromatograph
HUFA	Highly unsaturated fatty acid
K	Fulton's condition factor
LDL	Low-density lipoprotein
MAS	Motile Aeromonas species
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MUFA	Monounsaturated fatty acid
NFE	Nitrogen-free extract
PG	Plasma glucose
PER	Protein efficiency ratio
PI	Protein intake
PLT	Platelets
PPV	Protein productive value
PUFA	Polyunsaturated fatty acid
RBA	Respiratory burst activity
RBC	Red blood cells
RGR	Relative growth rate
RDW	Red blood cell distribution width
ROS	Reactive oxygen species
RPS	Relative percent survival

SGR	Specific growth rate
SP	Serum protein
SR	Survival rate
TCL	Total cholesterol
TP	Total protein
Trig	Triglycerides
WBC	White blood cells
WG	Weight gain

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background to the study

Like other industries and farming activities, aquaculture is an important industry for food security (Sakai, 1999). The importance is drawn from the recent demand for the aquatic food products. This has brought about human intervention into the intensive production of fish. This demand arises from the increase in human population and the advantages derived from the available natural water bodies around the globe (Lahsen & Iddya, 2014). The world production of fish has shown a positive relationship with time. In total, the world aquaculture fish production accounted for 44.1% of both human consumption and non - food uses (Food and Agricultural Organisation (FAO), 2016).

Consequently, there are pending issues with feeding and nutrition over the years. According to Thomson and Amoroso (2011), the lack of proper diets containing protein, vitamins, energy, minerals in the body has been found common among the people living in the rural sector and particularly the developing countries. The consequence is the increase in child mortality, the mental risk to sickness and goitre. It was reported that over 800,000 children are dying per annum due to zinc deficiency while two billion people worldwide are vulnerable to the poor diet of iron deficiency (Thomson & Amoroso, 2011).

1.1.1 Global aquaculture production

At the global level of aquaculture production, many of the regions are producing at a lower rate. Since 1995, the growth trend of the aquaculture industry indicated that the Asia continent had maintained a higher level of output. The output for Asia in 1995 in tons was 21,677.5 which increased to 65,601.9 in 2014. Though the Asia aquaculture

production had been maintained compared to other continents, the Asia aquaculture industry has been 'production trapped' for a long period (Means that the annual production percentage increase, has been stable for a long period). Given the data provided by FAO (2016), the rate of production in 1995 and 2014 were 88.91 for the two periods. The global trend of aquaculture fish production in tons is shown in Figure 1.1.

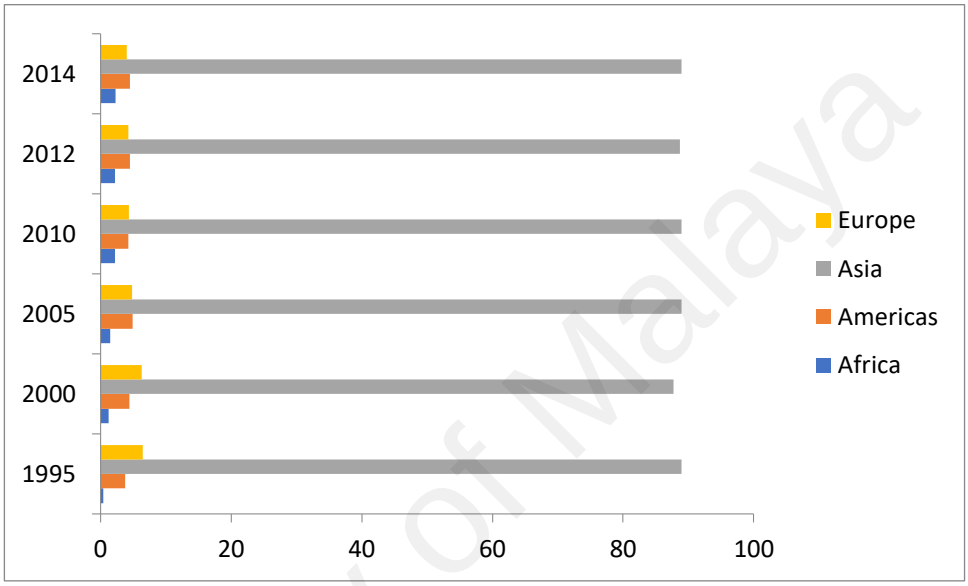


Figure 1.1: Global aquaculture fish production in tons (FAO, 2016)

From the Figure 1.1, it is observed that besides Asia that has the largest proportion, other regions have low output. The low output is manifested more in the continent of Africa. In addition, the production of wild fish globally has declined resulting in the diversion of attention to the aquaculture production technique. Currently, the aquaculture fish production has increased the proportion of fish consumption. On estimation, an average of 9.41kg was consumed per head in the household in 2012 (FAO, 2012). In the year 2012, the production of fish reached a peak of 66.63 million and widely consumed. Regarding the distribution across the household and boundaries, it has been unevenly distributed due to low income of the households and the poverty level of developing countries.

Basically, the low production was blamed on the type of feed applied by the fish farmers (FAO, 2016). Farmers are expected to produce at higher quantity depending on the supply and the quality of the feed available. According to FAO (2016), fish farmers are to be provided with a well-balanced feed that is cost effective with the purchase price of the feeds to reach an optimum level of profit. However, this has been constrained with poor policy leading to policy vulnerability and poor feeds application. Due to the prohibitive price of the feeds and poor policy, the African and Asian fish farmers have resorted to the application of aqua-feeds that are "either on-farm or by small-scale feed manufacturers" (FAO, 2016). This is aimed to improve the quality and the quantity of fish production and as well as maximizes a higher level of profit (FAO, 2016).

1.1.2 Aquaculture employment issues in Africa

The current data of the reporting institution showed two scenarios. First, the employment generation in the sector is slow for Africa over a period. Second, there was a decline in the number of fishermen in the fishing industry in 2014 (Figure 1.2). In 2013, there were 6009 fishermen in Africa but declined to 5,674 in 2014 (FAO, 2016). This could have resulted from different challenges facing the fishing farmers in the region (Moehl & Machena, 2001). These challenges range from government policy on fishing to inability to identify the right feed for optimal production (Sakai, 1999; Moehl & Machena, 2001; FAO, 2016).

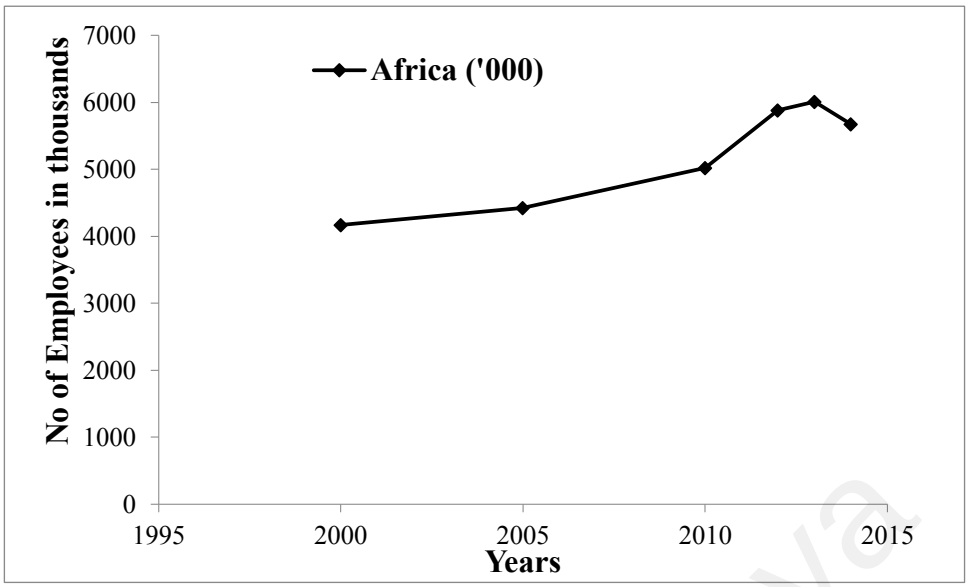


Figure 1.2: Africa fish employment ('000)

The employment in fishing for Africa shows a decreasing trend in 2014 which would influence the aquaculture industry and the consumption of fish products (Moehl & Machena, 2001). Another paradoxical issue was the employment of the fish farmers in Latin America and the Caribbean region compared with the level of fish production. The employment of the region showed an increasing trend, while production is low compared with other regions. Figure 1.3 below explains the employment trend of the Latin American fish farmers in thousands.

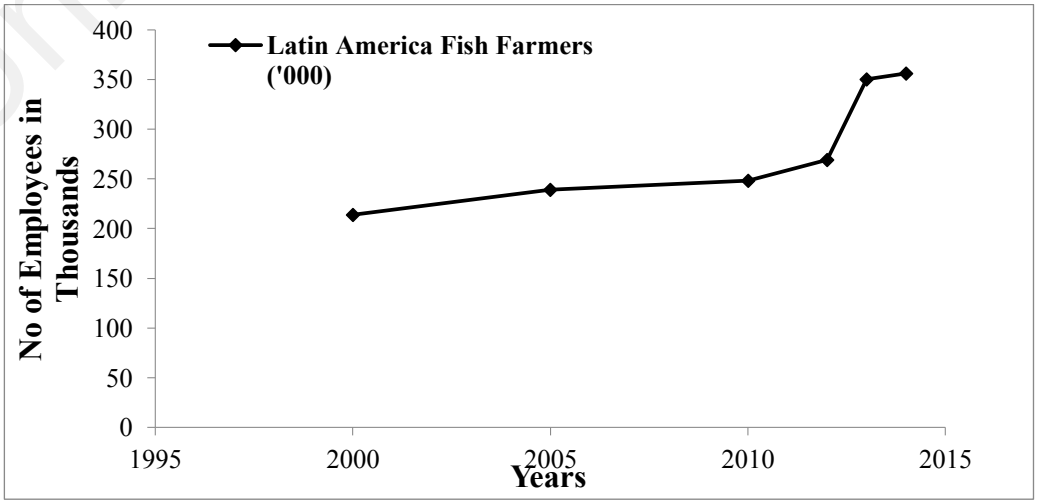


Figure 1.3: Latin America fish farmers ('000)

1.1.3 Need for fish production

The production and consumption of aqua-cultured fish are becoming cogent in human activities. According to Kris-Etherton *et al.* (2009), the cardiovascular disease killing hundreds of thousand globally can be curtailed with the consumption of fish that contains high-quality protein. Not only that fish contains protein, it equally possesses amino and fatty acid, minerals as a good fish meal for the teeming population. In this respect, it was confirmed that human dieting on fish products from natural waters has the capability to reduce all related heart diseases (FAO & WHO, 2010). Further studies show evidence that mothers consuming sufficient fish during pregnancy increase the possibility of neurodevelopment among the infant and young children (Thompson & Amoroso, 2011).

As noted that fish consumption is good for the body and to reduce mental and infant mortality, developing Africa, and Asia have embarked on fish production through aquaculture. For example, comparing Nigeria and Malaysia, it has been found that the two countries have fresh water for aquaculture production of fish (DOF, 2014). This has increased the volume of aquaculture production and consumption of fish in the two countries. Nevertheless, the two countries have challenges associated with feed supply and the cost of feeding the fish, particularly the African catfish. This has led some aquaculture farmers to quit the business. The increase in the cost of production led to the increase in the price of catfish. The higher the price of the product, the lesser the demand for catfish among the households. In consequence, there is a need for alternative aquaculture production protein sources to improve the quantity and quality of the product. This will bring the price of the product down to be affordable to the households.

In the quest to explore the alternative aquaculture production protein sources, Henry *et al.* (2015) suggested that plant protein would be a reliable source of amino acid

for feeding aquaculture catfish. The experts in fish nutrition had applied plant protein as a replacement for the Orthodox fishmeal (FM) diet in feeding catfish. They have incorporated variously required pulses and lupines like in the case of *Oncorhynchus mykiss*, *Salmo salar*, and *Dicentrachus labrax* (Carter & Hauler, 2000; Gouveia & Davies, 2000; Refstie *et al.*, 2000; Glencross *et al.*, 2004). Another suitable alternative feed for aquaculture is the soybean product which has its primary usage in the tropical omnivorous fish feed. This alternative use of soybean in fish feed has been reported by Fagbenro and Davies (2001) and Goda *et al.* (2007). The soybean formula for feeding fish is popular and efficient in aquaculture fish production. However, despite its acceptance, soybean,like cottonseed cake and other leguminous plants, apart from having anti-nutritional factors are also high in demand in human and animal nutrition.

The microalgae have been reported to contain high-quality protein (Phang *et al.*, 2000), amino acid characteristics when compared with other reference aquaculture food protein contents (Becker, 2007; Reyes-Becerril *et al.,* 2013), and also as food additives, colouring the flesh of Salmonids and for inducing other biological activities (Muller-Feuga and Richmond, 2004). As pointed out, microalgae also favoured polyunsaturated fatty acid, β-carotene, antioxidants, sulfated polysaccharides and sterols (Reddy *et al.*, 2000; Otles & Pire, 2001; Xue *et al.*, 2002).

Further studies showed that *Chlorella* and *Spirulina* are suitable as aquaculture feed for fish production (Becker, 2004; Becker, 2007). From the studies, the argument was based on the nutritional qualities such as high protein, amino and fatty acids. It also promotes antioxidant and immunostimulatory features.

1.2 Problem statement

Aquaculture has become a prominent means of food security in the global consumption. However, the production varied across continents. Although in inland aquaculture, Africa continent remains the second largest producer after Asia, in the production of marine aquaculture, however, Africa continent remains the lowest producer in 2014 (FAO, 2016).

In the Sub Saharan Africa continent, the production capacity of aquaculture was placed at a very low rate (FAO, 2016). The low production capacity in many regions of the world led to the scrutiny of different nutritional supplements and substitutes (plants and animals) to fishmeal in the feeding of different fish species, particularly the African catfish.

In the literature, there are debates about suitable fishmeal replacement on whether to apply *Chlorella* or *Spirulina* to increase the aquaculture production capacity (Becker, 2004; Becker, 2007). While some school of thought believes in microalgae protein as suitable alternatives Henry *et al.* (2015), others argued that soybean feed is appropriate (Fagbenro & Davies, 2001; Goda *et al.*, 2007). Another argument of microalgae as feed for increasing aquaculture production capacity is that it contains the required nutrients for fish survival (Bhat & Madyastha, 2000; Reddy *et al.*, 2000; Otles & Pire, 2001; Xue *et al.*, 2002). Based on different studies on these varieties of fish feed ingredients, it is observed that the debate on the best alternative to FM in aquaculture fish feeding is inconclusive.

Another issue to consider when choosing a suitable FM replacer is the location where the aquaculture industry is sited. Numerous studies showed that the aquaculture fishing farms are in the rural areas. These are managed by people with little education, as such, the farm could not cope with different bacterial diseases. This is due to

immunological therapy used by these aquaculture farmers. The effect of applying immunological therapy was reported by Sakai (1999), that the "immunological functions are associated with increased protection against infectious disease". Hence there is the need for feed that would not only serve the protein needs of the cultured fish species but also stimulate their innate immune response against pathogens in the aquatic environment.

Numerous species of algae farming had been tested, however, less than twenty of the species are recognized in the aquaculture industry. The microalgae species useful to nurse ornamental fish, prawns and salmonid fish are *Dunaliella salina*, *Haematococcus pluvialis,* and *Spirulina.* They served as natural sources of protein and principal producers of essential and highly high-quality omega-6 and 3 fatty acids like eicosapentaenoic acid (EPA), docosahexaenoic acid (DPA) and arachidonic acid (ARA) which are passed up the food chain to the fish in the aquatic environment (Henry, 2012).

Finally, there are other crucial factors to consider when choosing the nutritional ingredients (plant or animal) that would be used to feed fish: These factors include fish growth and health, the ability of the fish to digest the feed, the risk attributable to the feeding and how the fish immune system would adapt to the new feed (Sakai, 1999). Considering these criteria for fish health and optimal production of cultured fish, the study has chosen the African catfish species to determine the best feed leading to fish healthcare and optimal production.

1.3 Research questions

From the inconclusive debate on the aquaculture of fish diets, this thesis is determined to answer this pertinent research question.

i. What is the level of digestibility of *Spirulina* and *Chlorella* in comparison to fishmeal in African catfish?

ii. What effect do dietary *Spirulina* and *Chlorella* have on the growth and body composition of African catfish?

iii. Does the *Spirulina* and *Chlorella* meal have any significant impact on the immunity of the African catfish?

iv. To what extent does the *Spirulina* and *Chlorella* meal impact on the anti-oxidative response and Hematological profile of the African catfish?

1.4 Research objectives

The major research objective of this study is to examine the effect of *Spirulina* and *Chlorella* as an alternative to fishmeal in practical diets of *Clarias gariepinus* (African catfish). However, the specific focuses of the study are:

1. To examine the digestibility rate of dietary *Spirulina*, *Chlorella* and fishmeal nutrients amino and fatty acids in the African catfish.
2. To determine the replacement effect of fishmeal with *Spirulina* and *Chlorella* on the African catfish growth and body composition
3. To examine the impact of dietary *Spirulina* and *Chlorella* on African catfish immunity.
4. To assess the influence of *Spirulina* and *Chlorella* feeding on the African catfish' anti-oxidative enzymes response, haematological and biochemical profiles.

1.5 Hypothesis of the study

1. Dieting the African catfish with *Spirulina* and *Chlorella* does not increase the nutrient and amino acid digestion.
2. Replacement of orthodox fishmeal with *Spirulina* and *Chlorella* has no negative effect on the growth of African catfish.
3. Replacement of *Spirulina* and *Chlorella* improves the African catfish immunity and the antioxidant enzyme activity.

1.6 The contribution of the study

A lot of work has been carried out in aquaculture that established the kinds of aqua-feeds that could be used by the fish farmers especially in Africa. However, little was purely accepted as the best. In consequence, this thesis contributes to the literature by verifying whether *Spirulina* and *Chlorella* are sufficient to partially replace FM in aquafeeds for farmers to improve African catfish production. The examination of the feeds and its implication on the fish immunity is another contribution, particularly to confirm the existing knowledge on the application of *Spirulina* and *Chlorella* (for the first time) feeds on African catfish.

1.7 The organisation of the thesis

This thesis used a conventional method of thesis writing and divided into six chapters.

- Chapter 1: The chapter provides background introduction regarding the focus of the thesis. It discusses the global aquaculture production and the need for fish production especially for the creation of employment and nutritional complementarity. It discusses the problem of the study and states the research questions and objectives which were achieved in the latter part of the thesis.

Four hypotheses are stated which are used to unravel if *Spirulina* and *Chlorella* can partially replace FM in the practical diets of the African catfish.

- Chapter 2: Chapter 2 constitutes the review of previous works as related to the four objectives of the thesis. Among others, it focused on the conventional feeding, the African catfish threshold, the nutrients, the previous methodology used and health as well as nutritional processes.
- Chapter 3: The methodology chapter deals with research procedures employed to achieve the stated objectives regarding research design, material and methods, experimental set-up and data analysis involving summary, organizing and estimations of the parameters.
- Chapter 4: This chapter of this thesis focuses on the presentation of the results for each of the research objectives/hypothesis. The chapter is subdivided into sections to capture each of the objectives stated in Chapter 1.
- Chapter 5: The chapter was designed to discuss the result of the findings got from statistical analysis in Chapter 4. For coherency, each of the objectives was discussed in relation with the previous studies.
- Chapter 6: The final chapter deals with the conclusion and recommendations based on the discussion of the findings in Chapter 5 of this thesis. Finally, the thesis provides the direction for further research regarding the feeding of African catfish.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

The aquaculture fish production is a food security issue. A lot of literature had been written regarding it. In so doing, the study considered and reviewed the related literature on aquaculture, nutritional practice, health issues, and nutritional measurement to ensure that there is an update of knowledge of the aquaculture fish production. So, the chapter is subdivided into ten sections.

2.2 Global assessment of aquaculture production

The production varies across the nations. The production from aquaculture depends on the perception and the readiness of the people to consume the product. It equally depends on the knowledge of its importance to the human body. As such, data shows that the output of fish production increased from 19% in 1985 to 44.1% in 2014 (FAO, 2016). Specifically, aquaculture production is concentrated in Asia compared with other continents. In Asia, it was reported that China aquaculture output was found to be 61.85% in 2012. It declined to 61.62% in 2014 (FAO, 2016). Other selected Asian countries have low output. For example, Thailand, India, Vietnam, Indonesia and Bangladesh that have a percentage of aquaculture output higher than 2% but not greater than 7.1%. Other Asian countries like Malaysia produced below 2% in 2012 (FAO, 2012).

Although the developed countries such as Norway America and Europe are doing well in other economic productions, they are poorly inefficient in the aquaculture industry. Unlike China that dominated the market with 61.62% of the total output, Norway, America, Southern Europe, and Oceania contributed 1.81%; 4.54%, 0.41%, and 0.26% respectively (FAO, 2016).

In developing economies, aquaculture falls under small-scale enterprise (SME). Aquaculture has the strong economic capacity that influences the earnings of the local entrepreneur. As reported by FAO (2006), aquaculture increases the fish stock and the stock ownership. This is due to its relation to the husbandry of aquatic species. The level of its profitability and the contribution to human health sustenance has resulted in increases in the agribusiness of aquaculture as seen in Nigeria (FAO, 2012).

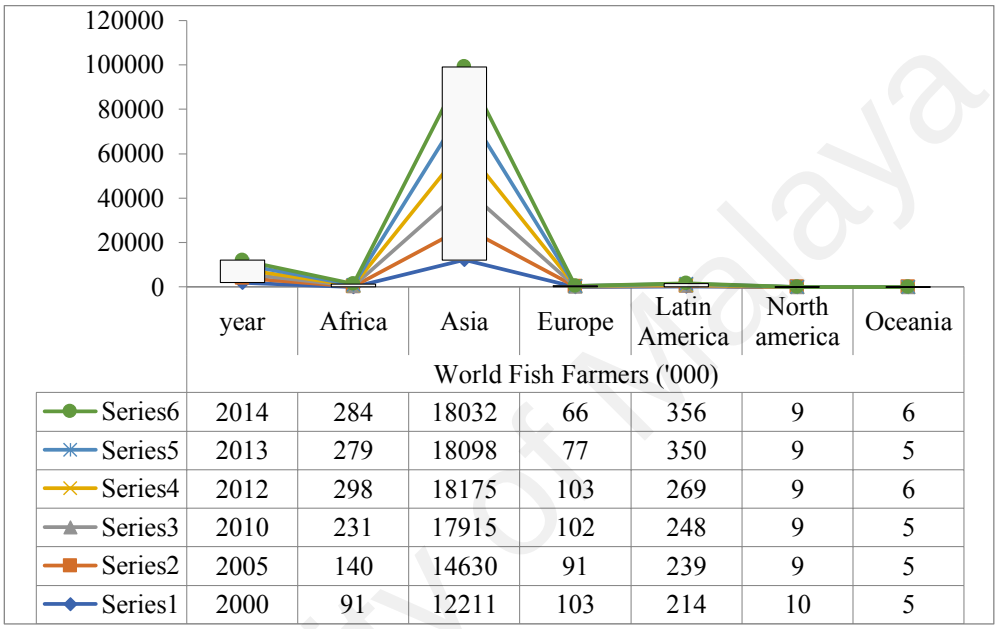
From employment driven by aquaculture, FAO (2012) reported that the aquaculture industry was most popular in the rural areas. This considerably increased job opportunities for the rural households. Specifically, FAO (2012) argued that an estimation of 16 million people was given employment through aquaculture. The opportunity arose from the demand for aquaculture fish products worldwide. In the aquaculture industry, there are different fish species. On one hand, FAO (2012) categorized these aquatic animal species into finfish, crustacean, molluscs, and others. On the other hand, there are aquaculture and mariculture. From these categories, finfish contributed 38,599 tons in inland aquaculture and 5,552 tons in mariculture. This gave 66.3% of the total production (FAO, 2012).

Table 2.1: Aquaculture world fishers by continents 2000-20014.

Year	Africa ('000)	Asia ('000)	Europe ('000)	Latin America (‘000)	North America (‘000)	Oceanic (‘000)
2000	4175	39646	779	1774	346	126
2005	4430	43926	705	1907	329	122
2010	5027	49345	662	2185	324	124
2012	5885	49040	647	2251	323	127
2013	6009	47662	305	2433	325	47
2014	5674	47730	413	2444	325	46

Source: (FAO, 2016)

From the table, it is displayed that Asia is leading in using aquaculture fishers in providing employment across the population while Africa remains the second in using aquaculture farming for generating employment. The trend of World fish farmers in respect of Africa and other continents aquaculture employment status is shown in Figure 2.1 below.



	year	Africa	Asia	Europe	Latin America	North america	Oceania
	World Fish Farmers ('000)						
Series6	2014	284	18032	66	356	9	6
Series5	2013	279	18098	77	350	9	5
Series4	2012	298	18175	103	269	9	6
Series3	2010	231	17915	102	248	9	5
Series2	2005	140	14630	91	239	9	5
Series1	2000	91	12211	103	214	10	5

Figure 2.1: trend of world fish farmers in respect of Africa aquaculture employment status 2010 – 2014

Assessing other developing economies, Malaysia contributes its little share in the production of aquaculture fish farming. Specifically, the commercial production of African catfish in Malaysia began in 2000. This was started with artificial mass seed production (Tuan *et al.*, 2003). Currently, freshwater and brackish water are where aquaculture thrives in Malaysia. Due to the discovery of better advantage of brackish water over fresh water, brackish water fish farming has gained a prominent position among the farmers. The brackish water has a total output of 414,000 while fresh water produced a total of 107,000 tons per annum (DOF, 2014). Abdel-Warith (2002), argued

that the increase of the output was due to the higher demand for local consumption of aquaculture fish (Abdel-Warith, 2002; DOF, 2014).

It was equally argued that through the freshwater pond and concrete tank aquaculture fish production in Malaysia, employment had been provided with upward of 20,976 in 2003 alone FAO, (2003). Through the level of output from the aquaculture farming, Malaysia made a significant supply of protein for human consumption (FAO, 2008). It has made a significant impact on poverty reduction programme of the Malaysian government. Nevertheless, the catfish production dropped in 2014, in approximation, 46,000 tons (DOF, 2014), given a total percentage drop of 0.08% to 2013 total output of catfish. In a nutshell, DOF (2014) asserted that African catfish and other aquaculture commodities have created an economic solution for agriculture-based countries such as Malaysia (Taufek, 2016).

2.3 Aquaculture farming in Sub Saharan African

The emergence of aquaculture fish production in Sub Saharan Africa (SSA) was in 1946 in the Democratic Republic of Congo (Moehl & Machena, 2001; Vincke, 1995). Tilapia fish was successfully practised in DR, Congo in aquaculture farming. Thereafter, other countries in the continent began to enjoy farming different fish production with aquaculture technique (Moehl & Machena, 2001). As of 1998, the aquaculture farming output reached a total of 120,000m (Moehl & Machena, 2001). Specifically, in 2014, the total output for the aquaculture for the SSA has reached a peak of 241 thousand metric tons.

The output of aquaculture in Africa indicates a positive response to aquaculture growth. El-Naggar (2008), argued that in Egypt, there is a high demand for aquaculture products. Prior to 1990, a large amount of 200,000 tons of fish were imported into Egypt

due to high demand for aquaculture products. Hence, the output increased from 35,000 in 1992 to 540,000 in 2005. This gave 93.52% rate of increase in output during the period. Considering population increase, El-Naggar (2008) anticipated for higher supply and demand for aquaculture products in subsequent years. Specifically, it was projected that the aquaculture industry in Egypt would be producing about 1.5 million tons in 2017. This made the aquaculture industry more prominent in Egypt (El-Naggar, 2008).

Aside from Egypt that maintains larger production in Africa, Nigeria remains the largest producer of Aquaculture fish product (Moehl & Machena, 2001; Hecht, 2007) in the SSA. It was argued that over 80% of the producers are small-scale farmers (Hecht, 2007). The other countries of the SSA have a low tonnage of the output. This is a challenge for the region. The challenges arise from the economic incapability of the rural farmers (Moehl & Machena, 2001; Nguenga *et al.*, 2008). The countries that largely embark on the aquaculture farming are Nigeria, Côte d'Ivoire, Zimbabwe, Kenya, Cameroon and South Africa (Moehl & Machena, 2001; Nguenga *et al.*, 2008). For example, Cameroon has an aqua water-friendly environment to produce a large quantity of fish. However, they are constrained by institutional policies either national or international such as the International Monetary fund (IMF) policy (Nguenga *et al.*, 2008). These challenges are responsible for the low output experienced in the SSA (Moehl & Machena, 2001).

2.4 African Catfish – Biological explanation

The African catfish *Clarias gariepinus* has become more popular among world fish farmers (Fleuren, 2008). Its origin was found in Africa which has transcended beyond subsistence to commercialized products (Adewolu *et al.*, 2008; Çek &Yilmaz, 2009). Not only is it widely farmed in Africa, the farming of *Clarias gariepinus* had been a popular industry in the Netherlands demonstrating its importance to human consumption as well

as nonconsumption factors (Fleuren, 2008). The *Clarias gariepinus* is produced through hybrid that leads to fingerlings. While examining the physical aspect of the African catfish, Fleuren (2008) argued that it is difficult to get the real identity of the catfish, control inbreeding, and monitored the genetic variation. He further argued that the farmers of African catfish could maintain the aquaculture through "electronic tagging, optimization of husbandry systems, and appropriate feeding regime and nutrition" (Fleuren, 2008).

The African catfish is an air-breathing fish among fish species. It is scale-less, with a long dorsal body and anal fin. Skelton (2001) explained that the body colour varies from dark to light brown with oily and greyish shades. Another school of thought argued that the African catfish accommodates low oxygen for survival. It uses a special suprabranchial organ for determining its time out in the water (Safriel & Bruton, 1984). In addition, it possesses the ability to tolerate harsh conditions in the moist sand and burrow environment. Such an environment contains an air-water vacuum for it to survive (Van der Waal, 1998). The life development of catfish is rapid with a space of time, both in weight and length (Britz & Pienaar, 1992). Earlier researchers stressed that African fish survives in high densities environment, hardy,prolific and easy to produce in captivity (Hecht & Appelbaum, 1987; Haylor, 1991; Toko *et al.*, 2007).

2.5 African Catfish (*Clarias gariepinus*) feeding methods

In the aquaculture industry, there are different feeding models that had been applied to African catfish *Clarias gariepinus*.

2.5.1 Conventional feeding

In practice, there are two modes of feeding African catfish, *Clarias gariepinus*. These include the pelleted feed and farm-made feeds (Nguyen & Oanh, 2009; Phan *et al.*,

2009). The pelleted feed contained fishmeal which consists of the required protein ingredients for the feeding of the fish. The protein ingredients are formulated per nutritional needs. The other mode, farm-made feeds, is a combination of the fish wastes and other animals. The procedure is to formulate the trashes to contain the required nutrients that would be sufficient for the catfish. This mode is widely used among African catfish farmers. It was argued that the two modes are expensive to procure in term of sources and monetary terms. Specifically, FAO (2002) reported that cost is a significant issue in keeping high production level in the aquaculture industry. Realizing this cost constraint, it becomes an issue in this thesis particularly to determine the appropriate feed for the African catfish at the lowest cost.

Several studies had been carried out on the African catfish (Jantrarotai *et al.*, 1994; FAO, 2004; Brummett, 2008; Fleuren, 2008; Phonekhampheng, 2008; Gordon *et al.*, 2013). From the studies, African catfish is an omnivorous animal that feeds on both plant and animals nonetheless are more adapted to animal-based diets. It is easier for African catfish to digest carbohydrate components easily when compared with other species of fish. While considering fishmeal for feeding African catfish, it was argued that fishmeal product is good for nursing African catfish. In addition to fishmeal, a combination of groundnut, rice bran, cotton seed cake and blood meal has been found to be effective in their developmental stages and growth (Abdel-Warith, 2002).

2.5.2 Other methods of feeding African Catfish (*Clarias gariepinus*)

There other non-conventional model used ingredients that will yield a maximum level of output over time. Among them is hydrolysed feather meal (Madu & Ufodike, 2004), toad meal (Ayinla, 2007), rumen epithelial meal (Sotolu & Adejumoh, 2008) and pigeon pea meal (Ogunji *et al.*, 2008). These non-conventional modes were reported to be more efficient in feeding the catfish. For example, (Phonekhampheng *et al.*, 2009)

reported that if the golden apple snail, *Pomacea canaliculata*, is replaced with the fishmeal, it increased the growth performance of the African catfish, with no negative effect on the feeding efficiency.

2.6 African Catfish nutrition threshold

This section provides detail requirements of how African catfish is nursed with available feeds by the nutritionist. The study discusses amino acid, protein, carbohydrate and fibre, lipid, energy, vitamins, minerals, and trace.

2.6.1 Protein threshold

In all living things, either aquatic animals or land animals, living appreciation depends on the quality of intake. The African catfish is not an exception. As earlier mentioned, the aquatic animals are either herbivorous, carnivorous or omnivorous. The African catfish is an omnivorous aquatic animal whose feeds encompass protein for its growth and development. This is because fish generally requires sufficient protein to grow if compared with terrestrial animals due to energy consumed while in water (National Research Council (NRC), 1983). However, the African catfish has the threshold of protein that it consumes for its growth. Relating to some studies on African catfish, lower dietary protein is the benchmark for its survival. From the work of Lucas and Southgate (2012), feed that contains protein, carbohydrate and lipid are not only cost-effective for the farmers but will satisfy the energy required by the catfish to grow. Feed that contains high protein level is vulnerable to leakage of nitrogen into the environment and a waste of the feed Li *et al.* (2006).

While providing the measurement of protein to feed catfish, (Li *et al.,* 2006) argued that the threshold of crude protein should be 24%. The 24% of the crude protein is sufficient to feed the catfish efficiently and sustain the catfish growth with a similar

outcome as the traditional, 32%-35% Fed to channel catfish (*Ictalurus punctatus*). However, if crude protein would be applied, it is recommended that 28% is sufficient to avoid wastage. The 28% would be applied to less than 10,000 individuals (fish) per acre. The daily feeding is placed at 80 pounds per acre. With this threshold, the quantity of the feed would enhance growth and moderate the lipid content consumed with an attendant better-quality yield. However, Cho *et al.* (1985) positioned that if the feed crude protein exceeds 45%, the consequence of catfish growth would be affected negatively. It will depress the growth if compared to when the fish farmer applies a lower crude protein percentage, ranging from 25% to 30% (Cho *et al.,* 1985).

The application of the crude protein also depends on time and the stage of the catfish. The NRC (1993) supported that the crude protein should be applied according to the fish life stages. The stages are in four phases. These include a fry, juvenile, grow out and broodstock stages. Each stage dictates the quantity that the fish farmer would use the crude protein to feed the catfish. It is expected that the aquaculture farmers feed the catfish as detailed in Table 2:2.

Table 2.2: Crude protein fish feeding threshold

Fry Stage	**Juvenile Stage**	**Grow out Stage**	**Broodstock Stages**
40% to 50%	36 - 40%	25 - 36%	25 - 36%

Source: (Li *et al.*, 2006)

In contrary, the aquaculture farmer avoids following the quantity at each of the stages. The aquaculture farmers have been feeding with irregular pattern outside the conventional protein threshold as stipulated in the work of (Li *et al.*, 2006)

Meanwhile, at the bloodstock stage, Quintero *et al.* (2011) argued that even though channel catfish Fed dietary protein of 32 – 42% improves eggs size and biochemical components, there were no significant effects on spawning, and fertilization compared to the lower crude protein applied by the aquaculture farmers.

2.6.2 Amino acid threshold

To make the catfish grow rapidly, the amino acid threshold must be met. There are ten essential amino acids (EAA) required for all vertebrate animals to grow physically. They are tryptophan (Trp), arginine (Arg), phenylalanine (Phe), isoleucine (ile), histidine (his), threonine (Thr), leucine (Leu), valine (Val), methionine (met), and lysine (Lys). However, these ten-amino acids requirements are sourced from external sources because the catfish cannot self-synthesize the nutrients. Considering the importance of amino acid in the growth and development of the catfish, Lucas and Southgate (2012) argued that the amino acid threshold is more important than the crude protein threshold, though required. The Higher threshold is required from amino acid while lower crude protein threshold is required for the physical development of the African catfish.

Feeding African catfish, the nutritionist does consider a lot of issues of dietary conditions. For example, Wu (2013) argued that environmental factor, dietary components, the pathological status of the fish and the physiological needs are issues that the farmers should consider. In a common aquaculture industry, the most limiting EAAs in typical fish diets are methionine and lysine. To improve the deficiency of essential amino acid required, introducing a non-essential amino acid would be sufficient. Rather adhere to feeding the fish with methionine; replacing it with cysteine would cut off about 65% of the methionine. Phenylalanine threshold can be replaced with Tyrosine. It will spare above 50% of the amino nutrients. While we consider the methionine and phenylalanine replacement, lysine is irreplaceable (Lovell, 1989; Li *et al.*, 2006). In

consequence, Li *et al.* (2006) argued that lysine should be introduced into the diet as a supplement to meet the amino acid threshold.

Table 2.3: Essential amino acid threshold of various fish species estimations

	Essential amino acids (g $100g^{-1}$ crude protein)									
Fish	Arg	His	Iso	Leu	Lys	Met	Phe	Thr	Trp	Val
Channel catfish[l]	4.3	1.5	2.6	3.5	5.1	2.3	2.1	2.2	0.5	3.0
African catfish [b]	3.6	1.2	2.0	3.5	4.8	2.4	4.0	2.8	-	2.4
Nile tilapia [a]	4.2	1.7	3.1	3.4	5.1	2.7	3.8	3.8	1.0	2.8
Common carp [a]	4.3	2.1	2.5	3.3	5.7	2.0	6.5	3.9	0.8	3.6
Rainbow trout [a]	4.2	1.2	2.8	2.9	5.3	1.9	2.0	2.6	0.4	3.4
Japanese eel[a]	4.2	2.0	3.8	4.7	5.1	4.8	5.8	3.8	1.1	3.8

Sources: [a] (NRC, 2011) [b] (Jimoh *et al.*, 2014)

The relevance of amino acid in feeding the African catfish is that it acts as precursors for the neurotransmitters, cofactors, and hormones. It is a vital aid in protein synthesis and metabolic reactions (Rodehutscord *et al.*, 1997; NRC, 2011). As such, the deficiency of amino acid in catfish feeding could lead to malfunctioning of the catfish physical system. Specifically, it leads to anatomical malfunctioning and yields poor growth. While considering the effect of deficiency of methionine, Page *et al.* (1978), Cowey and Sivak (1992) opined that insufficient nutrients have a significant effect on rainbow trout and lake trout. The effects are diminishing growth and lens cataract. Another study identified that nitric oxide that is used to control macrophage is produced with the arginine nutrient playing a significant role. The insufficiency of arginine amino acid has caused an increase in catfish death rate, suppress growth and eroding fin in the rainbow trout (Cho *et al.*, 1992) and channel catfish (Robinson *et al.*, 1981; Buentello & Gatlin III, 2001). One advantage that catfish has over the rainbow trout and sockeye

salmon is that it is not affected by scoliosis and lordosis infection which is caused by lack of tryptophan (Wilson *et al.*, 1978; Poston & Rumsey, 1983).

2.6.3 The lipid threshold

Lipid has been generally identified as a source of essential fatty acid (EFA) that are required in animal metabolism, according to Steffens (1996), it is an indispensable energy basis for providing linolenic (n-3) and linoleic (n-6) EFAs. Linolenic acid (n-3) fatty acid cannot be synthesized by freshwater fish, hence it must be incorporated into the diets. However, the fish farmers should adhere strictly to the formulated diet of the lipid (Li *et al.*, 1994). The amount of the lipid required often goes with the cost involved in producing the lipid fatty acid, fillet quality, and the conventional EFA threshold as well as producers' constraints (Li *et al.*, 1994). In the fish enzymatic process, Craig and Helfrich (2009) reported that DHA, HUFA, and EFA are produced which increases cellular membrane and other metabolic components.

The application of lipid nutrient requires that appropriate lipid contents are ensured. As argued by Yan *et al.*(2015), application of higher lipid nutrient in the fish feed would lead to excessive deposition of fat in the liver leading to health complications and reduce the market value. On the other hand, Li *et al.* (1994) positioned that the higher inclusion of linolenic (n-3) in the channel catfish diet particularly at a hot temperature, would affect the catfish immune system and reduce resistance against disease. Having those arguments, the conventional and practical diet for African catfish was explained in the work of Li *et al.* (2006). They recommended that catfish only requires 0.5% to 0.75% linolenic (n-3) fatty acid as well as lipid level below 5%. Comparing this with the NRC (2011) requirement, the protein energy for catfish is expensive. As such, increasing the lipid dietary level from 15% as against the recommendation of Li *et al.* (2006) of 5%

would reduce the cost of the diet and spare protein. With the 15%, lipid threshold would make the farmer reduce the level of protein in the diet without health issues (NRC, 2011).

2.6.4 Carbohydrate and fibre threshold

Comparing all the sources of energies for animals, carbohydrate forms the least among the nutrients for energy production. As least efficient, it is an unavoidable energy in physical growth of the animals. For instance, the African catfish, Nile tilapia, Pangasius catfish, channel catfish and Channel catfish belongs to omnivorous-warm water species. Carbohydrate nutrient in the diet is an essential source of energy better than the lipid nutrient in the diet (Wilson, 1994; Hung *et al.*, 2003). This is because the carbohydrate nutrient is stored in fish tissues and muscle as glycogen. During a shortage of food or other unfavourable situation such as hypoxic condition and high stock density, the carbohydrate will be a source of energy to the fish (Wendelaar Bonga, 1997). Nevertheless, the farmers should consider the carbohydrate requirements. Studies showed that dietary inclusion level, the complexity of the molecule, processing treatment, objective of nursing the catfish and carbohydrate source are the issues to consider in application of carbohydrate nutrient in the diet (Wilson, 1994; Stone, 2003; Enes *et al.*, 2009; Krogdahl *et al.*, 2010). For instance, suppose the aim of nursing the African catfish is market focused, Wilson and Poe (1985), in clear terms, emphasized the threshold for carbohydrate in the diet should contain wheat grain, cornmeal, and rice bran. These are feedstuff rich in starch content.

Other studies have demonstrated varieties of the threshold for carbohydrate nutrients in catfish feeds. For instance, in Jantrarotai *et al.* (1994) and Pantazis (2005), hybrid catfish (*C. macrocephalus* × *C. gariepinus*) tolerates semi-purified feed with a threshold of 50% carbohydrate while African catfish can tolerate 32% carbohydrate feed. Jafri (1998) drew attention to 25% carbohydrate with less than 4% fibre as feed threshold

for catfish. The recent contrary argument is that overfeeding the striped catfish with an elevated level of carbohydrate could lead to fish poor growth and size leading to poor marketing (Hien *et al.*, 2010). Also, it is sufficient for the fish with a lower fibre or cellulose to grow because the fibre leads to poor digestive process in fish digestion system (Li *et al.*, 2006).

2.6.5 Energy threshold

For all the animals, energy is required for physiological growth. The aquaculture energy threshold depends on the temperature of the water and the physiological condition of the fish (Guillaume, 2001). Calculating the energy required by African catfish, Henken *et al.* (1986) showed that 19 and 14 kJ kg^{-1} are required for African catfish in gross and digestive energies respectively. Examining the protein and energy ratio, 27 mg kg^{-1} is expected for the threshold. Nevertheless, this threshold holds with the level of the temperature. As the level increases from 24°C with the threshold of 25.4 mg kJ^{-1} to 29°C, then the threshold energy increases to 34.7 mg kJ^{-1}. To calculate the expected threshold, protein, lipids, and carbohydrates are the parameters for exact energy values in catfish formulated diet. These will produce physiological fuel for the catfish. As mentioned by Schulz *et al.* (2005), the averages for these parameters for the energy required in the catfish dietary formulation are 17.6 kJ/g for lipid, 23.9 kJ/g for protein and 39.8 kJ/g for carbohydrate. The threshold for the African catfish is that fish derives its energy from consumption of the formulated diet. However, there is a caution for the energy threshold. On one hand, the higher the energy acquired the less the feed intake and the insufficient nutrients leading to the poor growth of the fish. On the other hand, poor dietary energy formulated feed requirement for the catfish often lead to insufficient required nutrients leading to poor growth.

2.6.6 Vitamin and minerals

There are twelve vitamins nutrients required to feed African catfish. These include vitamin A, vitamin D, vitamin E, thiamine, riboflavin, pyridoxine, pantothenic acid (Wilson & Moreau, 1996), niacin (Kuczynski, 2002), folic acid choline, biotin (Mohamed *et al.*, 2000; Shaik Mohamed, 2001), ascorbic acid (Wilson & Moreau, 1996; Merchie *et al.*, 1997; Kuczynski, 2002; Datta & Kaviraj, 2003; Adewolu & Aro, 2009). The animal does not need a large amount of vitamin in its physiological development. Robinson *et al.* (2006), argued that animal growth, its health, and reproduction only depends on small quantities of organic compounds. Generally,the body produces vitamin for growth in which might not need a vitamin supplement. In the African catfish feed, the feed applied often contained an adequate quantity of vitamins and minerals to meet up with the catfish feed threshold. Equally, the feed supplied does make up for the losses from the feed processed and stored. So, the previous study indicated that the vitamin and minerals required for channel catfish are strongly recommended for the African catfish and other species (Wilson & Moreau, 1996). From the study, the threshold for the catfish diet which brought about true growth performance without negative signs because of poor vitamins defines the minimum threshold for a given requirement for feeding. Other studies such as Duncan *et al*. (1993) and (NRC, 1993) identified the negative signs that poor vitamins could bring. These include anaemia for deficiency of vitamin B12 and folic acid; oedema and exophthalmia signs due to deficiency of vitamin A in the African catfish. Considering the minerals for catfish feed, Robinson *et al.* (2006) argued that the catfish need mineral for metabolism and bone development. It also needs minerals for balancing the body fluids and the environment. These minerals are derived from the body of the water. Robinson *et al.* (2006) identified 14 minerals essential good for nursing African catfish.

Minerals are needed for fish metabolism and bone development besides balancing between body fluids and their environment. Some of the minerals can be absorbed directly from the water. However, there are seventeen minerals that are considered as being essential in catfish diets (Uys, 1989; Wilson & Moreau, 1996). These include calcium, phosphorus, magnesium, sodium, potassium, iron, Sulphur chlorine copper, manganese, zinc, cobalt, selenium, iodine, molybdenum, chromium and fluorine (Uys, 1989; Wilson & Moreau, 1996). Like vitamins, catfish feeds are typically supplemented with trace mineral premix and adequate supply of all essential minerals to meet the requirement of catfish (Robinson *et al.*, 2006). Table 2.4 shows the nutritional requirements of catfish.

Table 2.4: Nutritional requirement of catfish

Catfish species	**Nutrients**	**Recommended level**	**Major source**	**References**
African catfish	Protein	35 - 40%	Fishmeal	(Giri *et al*., 2003)
		35%	Casein + Gelatine	(Farhat &Khan, 2011)
	Lipid	> 8%	Palm oil	(Lim *et al*., 2001)
		10%	Sunflower oil	(Hoffman &Prinsloo, 1995)
	Digestible energy	18.56 kJ g-1	Casein, Dextrin, fish oil	(Pantazis, 2005),
Channel catfish	Protein	26 -32%	Various source	(Robinson *et al*., 2006)
	Lipid	4-6 %		
	Digestible energy	8.5 -9.5 kcal/g protein		
Asian catfish	Protein	36%	Fishmeal, Soybean	(Singh *et al*., 2012)
	Lipid	8%	Cod liver oil,	(Jafri, 1998)
	Digestible energy	14.1 MJ kg-1	Corn oil	

2.7 Fishmeal and aquaculture farming

Among the ingredients use in catfish formulated fish feed, fishmeal is commonly used in the aquaculture farming. It contains protein requirement in animal feed formulation. The common utilization of fishmeal in aquaculture farming was explained by (Sánchez-Muros *et al.*, 2014). As explained, using meat in fish feed production was legally prohibited. Putting into consideration the quality of fishmeal required for catfish growth, there are various conditions to work on. These include the drying, the raw fresh fish, and temperature of cooking of the fishmeal. However, cooking and drying the fishmeal with low temperature will increase the quality of the meal (Anderson *et al.*, 1993). This type of fishmeal production is known as low-temperature fishmeal (LTF) which is subject to second stage standard of fishmeal production in aquaculture catfish farming.

2.7.1 Types of fishmeal

In the previous studies, Ariyawansa (2000) classified fishmeal production into three. The first classification is on the fish caught primarily to produce fishmeal. Hence, it is not suitable for human consumption due to the concentration of oil and bones. Smelts, sardines, anchovies, and menhaden fall into this classification (Miles & Chapman, 2015). The essential of this fishmeal classification is that it usually contains higher quality basic nutrients required for growth such as balanced fatty acids and amino acids. It usually contains sixty-five percent crude proteins. The cost implication to produce this classification meal ranges from $385.00 to $554.00/tons (Miles & Chapman, 2015). It has large-scale production cost feature. Comparing the cost of this classification fishmeal with its counterpart soybean, it is higher with 2.5 percent to a range of 3.5 percent. The implication is that the aquaculture farmer would find it difficult to produce with cash-constraints (Miles & Chapman, 2015).

The second classification of fishmeal feed is named as trash fish or by-catch fish. According to Ramalingam *et al.* (2014), the bycatch fish often used are the short mackerel (*Rastrelliger brachisoma*), goatfish (*Upeneus* spp) and silver belly (*Leiognathus bindus*) in the production of fishmeal. It has a high concentration of protein. However, the problem associated with it is the poor storage facility because the protein declines with time if the by-catch fish is not properly stored in ice or chilled water prior to processing (Edwards *et al.,* 2004). Therefore, the by-catch fish in preparing fishmeal is often limited to small and medium catfish farmers due to storability problem.

The final classification as identified by Ariyawansa (2000) is the fish offal. The fish offal happens to be from the consumption firms. In this instance, the tissue, bone and the protein content are minimal than the entire fish. While comparing the offal fish with oily fish, Hempel (1993) stressed that a number of the nutrients such as the amino acid, the pilchard, and methionine, are considerably lower in fish offal. To improve the nutrient, he suggested that shrimp meal, scallop, and the crab are added during fishmeal production. This assures a better fishmeal to feed the catfish.

The global production of fishmeal in recent years is an issue for the aquaculture industry and farmers. FAO (2014) reported that the fishmeal production dropped considerably in 2013 as against 2012. The major producers are Chile and Peru. In 2013, the fishmeal production in these countries dropped to 799,000 tons. The limited production of the fishmeal resulted considerable in a hike in market prices of the feed thereby creating a low turnover among the small and medium aquaculture farmers. Those farmers who could not afford the hiked price applied low-quality fishmeal thus reducing the fish growth and market growth rate (FAO, 2014).

2.7.2 Health side effect of fishmeal

Although fishmeal is a common ingredient in feed formulation for African catfish production, it has its own disadvantages. Studies have shown that there are health effects when consuming fishmeal. In Easton *et al.* (2002), they identified bovine spongiform encephalopathy (BSE) health issue relating to fishmeal consumption. Another study by Jacobs *et al.* (2002) identified polychlorinated biphenyls (PCBs) contamination associated with the fishmeal consumption. The uncertainty of production, coupled with the low production tonnage of fishmeal, and the health hazards associated with its consumption necessitated finding its alternative replacement in African catfish feed.

2.8 Potentials of microalgae in aquaculture feeds

Macroalgae like the 'kelps' genus, *Undaria*, *Laminaria* and *Durvella*, and the brown algae, *Ascophyllum*, occur in dense stands, which can be harvested economically. They have a long history of use as iodine sources, soil amendment and animal feed additives to provide trace elements (Henry, 2012; Priyadarshani & Rath, 2012). Furthermore, microalgae farming that include *Haematococcus pluvialis Spirulina* and *Dunaliella salina* are natural feeds of salmonid fish, prawns and ornamental fish (Webb & Chu, 1983; Andersen & Kawachi, 2005; da Silva & Barbosa, 2009; Priyadarshani & Rath, 2012). *Dunaliella*, *Chlorella*, and *Spirulina* have been found to be the three major species of algae that have been successfully used to produce high concentrations of valuable compounds such as proteins, lipids, and pigments (Abe *et al.*, 1999; El-Behairy & El-Baroty, 2002; El Baz *et al.*, 2002)

The need for nutritional sources is more secure than the conventional fishmeal and other animal products have awakened interest in plants generally and algae specifically (Priyadarshani *et al.*, 2012). Microalgae perform an important role in

aquaculture. While plants are important to terrestrial animals, microalgae represent the natural biological process base, as well as the first supply of all the phytonutrients within the aquatic organic phenomenon (Webb & Chu, 1983; Chen, 2003; Priyadarshani & Rath, 2012). They are wealthy resources of nutrients such as essential fatty acid, amino acid, proteins, vitamins A, C, B1,B2,B6, and minerals like niacin, iodine, potassium, iron magnesium and calcium (Priyadarshani & Rath, 2012). They are also high in polyunsaturated fatty acids, β-carotene and antioxidants sulfated polysaccharides, pigments, and sterols for aquatic animals' (Reddy *et al.*, 2000; Otles & Pire, 2001; Takeuchi *et al.*, 2002; Xue *et al.*, 2002; Avagyan, 2008). Additionally, it has been shown that live zooplankton could be removed from the larval dieting of the Red drum if micro-algae were Fed a formulated microparticulate diet (Rocha *et al.*, 2008). Thus, it is not surprising that the biochemical composition of certain marine micro-algae is compatible with the alimental requisites of some marine fish (Henry, 2012). It is prominent that adding microalgae to larval fish rearing tanks confers many benefits, for example, averting bumping against the walls of the tanks (Battaglene & Cobcroft, 2007), enhancing predation (Rocha *et al.*, 2008), and nutritional values of zooplankton (van der Meeren *et al.*, 2007), as well as improving the immune and digestive functions of larvae (Cahu *et al.*, 1998; Spolaore *et al.*, 2006).

Microalgae are a characteristics part diet of numerous larval fish, either eaten directly or attained from the gut contents of prey species, for example, copepods and rotifers (Henry, 2012). Basically, the biological worth of an algal species for a specific organism is subject to its digestibility, size, biochemical composition, and production of toxic compound (Müller-Fuega, 2004; Priyadarshani & Rath, 2012). Since they are principal producers within the aquatic chain, microalgae supply many phytonutrients, as well as omega-3 fatty acid (DHA) and arachidonic acid (ARA) precursors of the treasured dietary aspects widely promoted as omega-3 fatty acids (Belarbi *et al.*, 2000; Griffiths &

Harrison, 2009; Harun *et al.*, 2010). However, the content of highly unsaturated fatty acids (HUFA), specifically omega-3 (EPA), docosahexaenoic DHA and arachidonic acid (ARA) is of major importance within the analysis of the biological process of composition of algae species to be used as food for marine organisms (Harun *et al.*, 2010; Rinna, 2014).

Microalgae feed is now a new trend in nursing zooplankton and feeding young animals (Chen, 2003; Priyadarshani *et al.*, 2012). Specifically, they pointed out that *Pavlova*, *Tetraselmis*, *Chaetoceros*, *Phaeodactylum*, *Isochrysis Nannochloropsis*, *Skeletonema Thalassiosira,* and *Chlorella* are the common microalgae in aquaculture feed. Among them, *Spirulina* and *Chlorella* are formulated for the feeding of some kinds of animals that include African catfish, poultry, and dogs (Priyadarshani & Rath, 2012). In the paper of Priyadarshani and Rath (2012), they stressed that *Nannochloropsis*, *Thalassiosira*, *Chaetoceros*, *Tetraselmis*, and *Isochrysis* as microalgae are species useful for larval feeds. Major producers of aquaculture feed have concentrated on the *Spirulina* and *Chlorella*.

Numerous species of macro and microalgae are incorporated into aquaculture feed formulations to assess their dietary worth (Priyadarshani & Rath, 2012). In many studies, they were found to be useful in a stress reaction, increasing growth, tolerating starvation physiological activities, feed utilization and quality of the carcass (Mustafa *et al.*, 1995; Gatlin, 2007; Güroy *et al.*, 2011). Patnaik *et al.* (2006), and Regunathan and Wesley (2006) also found that shrimp fish derives protein and carotenoid from the microalgae fish meal. Many species of algae have been used in different feeding trials involving fish, such as, *Undaria* and *Ascophyllum*, which was administered to Sea Bream (Yone *et al.*, 1986); *Ascophyllum*, *Porphyra*, *Spirulina*, in addition to *Ulva* Fed to Sea Bream (Mustafa *et al.*, 1995); *Gracilaria* and *Ulva* Fed to European Sea Bass (Valente *et*

al., 2006); *Ulva* Fed to stripped Mullet (Wassef *et al.*, 2001) *Ulva* and *Pteroladia* Fed to European sea bass and Gilthead Sea Bream (Wassef *et al.*, 2005; Wassef *et al.*, 2013); *Porphyria*, and a *Nannochloropsis -Isochrysis* mixture Fed to Atlantic Cod (Walker *et al.*, 2009; Walker & Berlinsky, 2011) *Ulva lactuca* Fed to *C.gariepinus*, white spotted snapper (Abdel-Warith *et al.*, 2016; Zhu *et al.*, 2016), as well as *Euglena*, Fed to rohu (Das *et al.*, 2009). Noteworthy, algae are chosen as fish feed for experimental purposes based on convenience and availability as well as an economic advantage (Henry, 2012). Both *Spirulina* and *Chlorella* may be cultivated in inexpensive open pond technology and marketed as dry powders. Their organic compositions are well-documented by many researchers to establish their dietary values and be used effectively in the aquafeed (Roy & Pal, 2015). From the work of Priyadarshani *et al.* (2012), the usefulness of microalgae is presented in Table 2.5 below.

Table 2.5: Uses of microalgae

Pigments/Carotenoids	**B-carotene, astaxanthin, lutein, zeaxanthin, canthaxanthin, chlorophyll, phycocyanin, phycoerythrin, fucoxanthin**
Polyunsaturated fatty acids (PUFAs)	DHA(C22:6), EPA(C20:5), ARA(C20:4), GAL(C18:3)
Vitamins	A, B1, B6, B12, C, E, biotin, riboflavin, nicotinic acid, pantothenate, folic acid
Antioxidants	Catalases, polyphenols, superoxide dismutase, tocopherols
Other	Antimicrobial, antifungal, antiviral agents, toxins, amino acids, proteins, sterols, MAAs for light protection.

Source: (Priyadarshani *et al.*, 2012)

2.8.1 Potentials of *Spirulina* and *Chlorella* in animal diets

Spirulina and *Chlorella* are commonly used in aquafeed due to their high contents of proteins with high biological value, indispensable amino and fatty acids, polysaccharides, antioxidant pigments in addition to their immunostimulatory properties (Phang *et al.*, 2000; Pugh *et al.*, 2001; Becker, 2004; Becker, 2007; Lordan *et al.*, 2011). *Spirulina* is a blue-green filamentous and photosynthetic Cyno-bacterium that grows in

fresh, salty as well as brackish water with high alkalinity (pH 10-12). It is very rich in protein (55-70%), with about 60% digestibility depending on the species and the sources (Phang *et al.*, 2000; Colla *et al.*, 2008; Ogbonda *et al.*, 2007; Lordan *et al.*, 2011) minerals, essential fatty acids (GLA), vitamins (essentially pro-vitamin A & B12), beta-carotene and phenolic compounds (Belay, 2002; Tokuşoglu & Uunal, 2003; Colla *et al.*, 2008; Gouveia *et al.*, 2008; Sajilata *et al.*, 2008; Dalle Zotte *et al.*, 2013) that are essential for growth. *Spirulina* is also rich in antioxidant pigments especially *Phycocyanin* and chlorophyll (Rangel-Yagui *et al.*, 2004; Madhyastha & Vatsala, 2007; Wang *et al.*, 2007; Capelli & Cysewski, 2010). Supercritical fluid extracts from *Spirulina platensis* show antioxidant and antimicrobial activity (Mendiola *et al.*, 2007).

In human nutrition, RNA, superoxide dismutase glycolipids, carotenoids, and DNA are other nutrients attributable to *Spirulina* food (Capelli & Cysewski, 2010). Comparing the *Spirulina* nutrients effect in the body, Capelli and Cysewski (2010) argued that *Spirulina* contains 180% of calcium than milk, 670% of protein than tofu, 3100% of beta-carotene than carrots, 5100% of iron than spinach, and higher anti-inflammatory and antioxidant than nutrients derived from vegetables and fruits. However, the analysis of Capelli and Cysewski (2010) are applicable to human food. Due to the presence of nutritional contents, it has been proposed as nutritional feed for the animal (Belay *et al*., 1993; Doreau *et al*., 2010; Holman & Malau-Aduli, 2013). The good advantages of the *Spirulina* prompted further experiment of applying it to fish feeding.

2.8.1.1 Potentials of *Spirulina* of fish diets

Numerous research work was directed to examine the influence of *Spirulina* alone or in comparison with other algae on development, immune responses as well nutrient utilization of different fish species including the work of Mustafa *et al.* (1997) on red sea

bream (*Pagrus major),* Nandeesha *et al.* (2001) on catla and rohu (*Catla catla* and *Labeo rohita),* Takeuchi *et al.* (2002), Ibrahem *et al.* (2013), Amer (2016), on Nile tilapia *(Oreochromis niloticus),* El-Sheekh *et al.* (2014) on hybrid red tilapia *(O.niloticus x O.mossambiqus),* Palmegiano *et al.* (2008) on Mekong giant catfish (*Pangasianodon gigas),* Tongsiri *et al.* (2010) on white sturgeon (*Acipenser transmontanus*), Kim *et al.* (2015) on olive flounder (*Paralichthys olivaceus*), Kim*, et al.* (2013) on parrotfish (*Oplegnathus fasciatus*), Yeganeh *et al.* (2015), Teimouri *et al.* (2013) and Sirakov *et al.* (2012) on rainbow trout, Dernekbasi *et al.* (2010) on Guppy fish, Ghaeni *et al.* (2011) on *Penaeus semisulcatus*, and the eminent works on *Cyprinus carpio* of Nandeesha *et al.*, (1998), Abdulrahman & Ameen (2013), and Abdulrahman & Ameen (2014). In addition to fish species, other researchers focused on the effect of *Spirulina* on crustaceans, including white shrimp *Litopenaeus schmitti* (Jaime-Ceballos *et al.*, 2005), pacific white shrimp (*Litopenaeus vannamei*) (Hanel *et al.*, 2007; Macias-Sancho *et al.*, 2014), *Spirulina*, *Chlorella* and *Azolla* on *Macrobrachium rosenbergii* (Radhakrishnan *et al.*, 2014).

Few researchers including Promya and Chitmanat (2011) and Sayed and Fawzy (2014) examined the effect of *Spirulina* on African catfish *(C. gariepinus)*, while Sayed and Fawzy (2014) worked on *Spirulina* alone, Promya and Chitmanat (2011) compared with *Cladophora*. These previous researchers, however, focuses on *Spirulina* as additives on the fish growth and welfare ignoring the effect of *Spirulina* on the oxidative stress enzymes. Thus, this thesis set out to use *Spirulina* as a partial replacement for fishmeal in the practical diets of the African catfish *(C. gariepinus)* to maximize its potential benefits as highlighted in Section 2.8.1 and Table 2.5.

Chlorella is a unicellular green alga that is extensively distributed in nature mostly in freshwater bodies and can survive by both heterotrophy and photoautotroph

using external carbon source (Yamaguchi, 1996). It is very rich in chlorophyll than any known plant, protein (51-58%) and essential nutrients like amino acids (Becker, 2007). It is also dense with polyunsaturated fatty acid, polysaccharides, pigments and added materials of important bioactivity (Han, *et al*., 2002, Kang & Sim, 2004). Over twenty vitamins and minerals are found in the algae as well as iron calcium, potassium, magnesium, provitamin A, vitamins C, B1, B2, B5, B6, B12, C, E and K, as well as biotin, inositol, and folic acid (Becker, 2004; Becker, 2007). Among its distinctive properties is the phytonutrient referred to as *Chlorella* Growth Factor (CGF), which is assumed to be focused within the nuclei. Befittingly, is comprised of nucleic acid related materials, proteins, amino acids, peptides, vitamins and sugars of specific interest with relevance to detoxification within the presence of peptide glutathione in the *Chlorella* growth factor (Nick, 2003). *Chlorella* according to Bengwayan *et al.* (2010) had a more robust activity in inhibiting lipid peroxidation as compared to glutathione and has antioxidant properties. It also contains β-1, 3-glucan, which provides strong immune stimulation, free-radical scavenging, as well as a reduction of blood fat (Spolaore *et al.*, 2006).

2.8.1.2 Potentials of *Chlorella* in fish diets

Chlorella like *Spirulina* has been investigated either as sole or in comparison with other algae species as feed additives or fishmeal replacements for growing, nutrient uses in addition to immune-boosting of different fish species. For example, the work of Bai *et al.* (2001) and Cho *et al.* (2001) on juveniles and larvae of Korean rockfish (*Sebastes schlegeli)*, Kim *et al.* (2002) on olive flounder (*Paralichthys olivaceus*), Pradhan and Das (2015) on rohu (*Labeo rohita*) and on gibel cap (*Carassius auratus*) (Xu *et al.*, 2014; Zhang *et al.*, 2014). *Chlorella* has also been used in comparison with other algae such as *Chlorella* with *Scenedesmus* on Nile tilapia (*Oreochromis niloticus*) (Badwy *et al.*, 2008), *Chlorella* and *Spirulina* with *schizochytrium* sp. on Nile tilapia (Sarker *et al.*, 2016). Kim

et al. (2002) reported a significant weight increase, better protein efficiency ratio and feed efficiency in juvenile Japanese Flounders (*Paralichthys olivaceus)* Fed diets supplemented with 2% *Chlorella ellipsoidal*. Body fat and serum cholesterol were also considerably reduced leading to improved lipid absorption. Badwy *et al.* (2008) reported better growth parameters by replacing FM protein with 50% of *Chlorella* and *Scenedesmus* (separately) in Nile tilapia diets. *Chlorella* carotenoid content was also found to enhance pigment in fishes (Gupta *et al.*, 2007). To the best of the author's knowledge and based on literature search, a study on the effect of *Chlorella* on African Catfish is little or nonexistent. Thus, in this thesis *Chlorella* in comparison with *Spirulina* have been utilized for the aforementioned advantages and as discussed in Section 2.8 and Table 2.5. Noteworthy, algae are chosen as fish feed for experimental purposes based on convenience and availability as well as an economic advantage (Henry, 2012). Both *Spirulina* and *Chlorella* may be cultivated in an inexpensive in open pond technologies and marketed as a dry powder. *Spirulina* can also be cultivated in sago waste (Phang *et al.*, 2000). Due to the high protein content and other nutrients, testing the *Spirulina* and *Chlorella* feeds in replacement with the conventional fishmeal in aquaculture feed in African catfish is essential.

2.9 Digestibility of feed by fish

Feeding of catfish goes with an examination of how digestible the feeds are applied to the fish. Measuring the digestibility enables the farmer or nutritionist to determine how the feed could be formulated and applied at a given time. Glencross *et al.* (2007) suggested four basic and indispensable factors that are to be considered when measuring the digestive value of nutrients in any feed ingredient. These include feeding approaches, the method by which faeces are collected, formulation of the diet and digestibility measurement. In another study by Hepher (1988), explained that three core

factors influence the fish digestion method. First, the food consumed and its level to which it is affected by the impact of digestive enzymes. Second, the activity of the digestive enzymes and finally the duration of the feed on the fish that makes it vulnerable to the action of the digestive enzymes.

2.9.1 Methodology for evaluating digestion issue in fish as application to African catfish

Conventionally, there are two methodologies often used to evaluate the applied feed and the digestive ability of a fish. In Lovell (1989), these are a direct method which is quantitative in measurement and indirect method which is less stressful (Bureau & Cho, 1999). First, the direct measurement takes care of all the nutrients consumed by the fish (ingested) and all the faeces passed out (egested). The values are subjected to an equation as presented in equation 2.1.

$$E = \frac{\alpha - \beta}{\alpha} * 100 \qquad 2.1$$

Where E = Percentage of apparent digestibility, α = Quantity of nutrient ingested into the fish, β = Quantity of nutrient egested from the fish.

With this model 2.1, it is easier to obtain the accuracy of the digestibility that occurred. However, it requires a high level of patience to prevent leaking the main nutrients into the water. Other negative side effects of the method are that attempt to collect the faeces (egested) from the water through stripping, the fish will be subjected to a lot of stress. A further effect of the method is that the stress exposed to would lead to poor digestive and metabolic process of the fish thereby producing inadequate results (Lovell, 1989; NRC, 2011)

The indirect measurement is often used by the fish farmers in digestibility evaluation. The method involves the collection of the excreted sample. It neither affected by the feed materials nor the use of the indigestible marker to assess the digestibility of the fish (NRC, 2011). In practice, the indigestible marker is either included in fish feed at a reduced concentration or be one of the feed components during formulation. Also, the kind of indigestible markers for fish meal formulation should not be toxic. These include titanium dioxide (TiO_2), chromic oxide (Cr_2O_3) and yttrium oxide (Y_2O_3) (NRC, 2011). From this method, digestibility is determined by the ratio of the indigestible marker (im) in the meal and faeces (f) that is:

$$DE = im / f \qquad 2.2$$

Where DE = *digestive evaluation* using *indirect method*
im = *indigestive* marker added to the feed
f = faeces passed out

From the above, the ratio of and explains the digestibility of diet applied and energy accrued by the fish. The merit of this method as explained by (Bureau & Cho, 1999) is the low stress for the fish because the faeces are collected in holding or rearing tanks.

The recent work has expanded the indirect method of evaluating the digestibility of the feed in fish farming. Goddard and McLean (2001) came up with a model they adapted from Cho *et al.* (1982) as specified below.

$$AD(\%) = [1 - (F / D * DM / FM)] * 100 \quad 2.3$$

where AD = Apparent digestibility

F = % of nutrient of energy in faeces

D = % nutrient of energy in diet

DM = % marker in diet

FM = % marker in faeces

To obtain the desired outcome, there are four assumptions to be met. The assumptions are: (i) the marker must be indigestible, (ii) the marker must be nontoxic, (iii) it should completely be inert, and (iv) they should move through the gut at the same rate of as the digesta (Goddard & McLean, 2001).

2.9.2 Empirical studies on digestibility

Several studies had been conducted to evaluate the digestibility of the nutrients such as protein amino acids, carbohydrates, and others. This thesis considered some of these empirical findings.

2.9.2.1 Protein and amino acid digestibility

In the work of Tonheim *et al.* (2007), the *in vitro* method was used to examine the digestibility of protein. They used the method to compare the digestibility in live and artificial larval feeds. Water-soluble nitrogen in the feed was analysed and the feed ingredients. From their analysis, the soluble reference protein (Na+-caseinate) has a higher digestible process than the insoluble reference protein (casein). However, their final digestibility was the same. The result of the *in vitro* digestibility was lower in larva formulated feeds than the frozen live feeds. It was discovered that the three focused meals of marine origin which include fish meal, squid meal and fish roe meal had low contents of water-soluble nitrogen (Tonheim *et al.*, 2007). However, the result showed different degrees of digestibility of the feed (Tonheim *et al.*, 2007). From the outcome of the study,

it is imperative to ascertain the exact bioavailability of dietary protein sources in larval fish (Tonheim *et al.*, 2007).

In McGoogan and Reigh (1996), it was discovered that the digestibility of protein is excellent in feedstuff with dietary protein greater than 60% and less than 2% in fibre contents. For channel catfish, the digestion coefficient for crude protein for high protein feedstuff is within the range of 75% to 95% (NRC, 1993). In Allan *et al.* (2000), the protein digestion of poultry offal meal, feather meal, blood meal and gluten meal were found to have apparent digestion coefficient (ADC) that ranges between 85 - 99% which compares favourably with fishmeal protein. Other findings showed that the CP in plant meal such as corn and wheat contains higher ADC than the nutrients derived from an animal such as feather meal as tested by Shahzad *et al.* (2006) in *Labeo rohita.* Considering the study of Fagbenro (1996) the ADC of feedstuff of plant and animal origin are similar in catfish. The study of Pantazis and Neofitou (2004) showed that the protein digestibility range was between 70% and 80% for fingerling of catfish that were Fed with algae and blood meal mix simultaneously. A recent study by Taufek *et al*. (2016) reported ADC of 81.21% as against 78.22% in juveniles of African catfish Fed cricket meal and fishmeal respectively.

2.9.2.2 Lipid digestibility

As explained in previous sections, fish needs lipid nutrient as a source of energy. (Robinson & Li, 2007; Li *et al.*, 2009) argued that lipid nutrient is an energy source for fish because it is easily digested by fish compared to carbohydrate. There are two factors for the lipid digestibility. These were stated by Hossain *et al.* (1992) as the water temperature and the dietary lipid. To ease the lipid digestibility of the fish, farmers should avoid increasing the ratio of the fatty acid particularly for the species belonging to cold and warm-water (NRC, 2011).

A recent study by Jimoh *et al.* (2014) indicated that for African catfish, the lipid ADC was approximately between 83% and 88% when sesame-based feed is applied. However, a different range was projected for channel catfish which ranges between 76% and 97%. Taufek *et al.* (2016) reported 89.82 % ADC lipid for African fish Fed cricket meal. An earlier study indicated that the result of animal and plant feed showed no difference in lipid digestibility (Hossain *et al.*, 1997; Mohanta *et al.*, 2006). Basically, Guillaume (2001) made a clear issue about lipid utilization. It is a species-specific. For example, in turbot, when lipid diet was increased to 15%, there was a reduction in growth performance and lipid ADC. Considering another species, a different result was obtained in trout and Atlantic salmon feed at a percentage higher than 30 of lipid diet. In this case, high lipid ADC and productive growth were observed.

2.9.2.3 Considering carbohydrate digestibility in fish diet

The starch element makes the feed least expensive no-protein energy for the African catfish because it is the cheapest and most abundant nonprotein energy source that is readily available in catfish diet (Abdel-Warith, 2002). Previous studies had found out that freshwater and warm-water make digestibility of high carbohydrate convenient for the catfish than nursing it in cold-water or marine (Stone, 2003). This result is based on the fact that the warm-water fishes contain a higher level of intestinal amylase. Experimental result of Cho and Slinger (1979) on comparing rainbow trout and channel catfish showed that channel catfish has the ability to digest up to 65% of carbohydrate (starch uncooked). In rainbow trout, its ability to digest the uncooked starch (carbohydrate) is below 50%. Considering the best starch required, Wilson and Poe (1985) suggested that rather than applying corn prepared using pellet mill, cooked corn is most efficient for the growth of channel catfish because it is digestible up to 38%.

From the previous experiment on carbohydrate digestibility in channel catfish, studies showed that attempt to increase the required carbohydrate diet reduce the capability of the catfish carbohydrate digestibility (Cruz, 1975). The components of carbohydrate such as corn, rice bran, and wheat grain enhance carbohydrate digestibility ranging between 60% and 70% in channel catfish (Cruz, 1975; Abdel-Warith, 2002).

Like in lipid digestibility, the carbohydrate digestibility is species-specific and feeding habits related. Grisdale-Helland and Helland (1998) pointed out that Atlantic halibut has poor toleration of elevated level of starch, as an increase of dietary carbohydrate from 8% to 17% resulted in a sharp decrease of digestibility value from 85% to 53%. As compared to carnivorous fishes, Krogdahl *et al.* (2005) pointed out that the herbivorous fishes can digest non-starch carbohydrates more efficiently. The sufficient gut microbiota present in herbivorous fish is responsible for this.

2.10 Health and nutritional processes in catfish maintenance

Like other animals, catfish requires a healthy life. In this section, the thesis explains different ways of administering nutrition to fish. This prevents negative health effects on both the fish and human (farmers).

Research has shown that nutrition plays an important role in the prevention of infectious diseases in man and animals. In the previous studies, specific areas such as nutrition, immune response, and the fish ability to resist disease were studied (Lovell, 1989; Barman *et al.*, 2013). With little understanding of the immune system of a fish, success recorded was small. From later evidence, the results to the health of nursing fish have a correlation with the dietary nutrition of the fish. In Lim & Webster (2001), the indicators that could have an effect on fish health were identified. These include feeding

regulation, nutrient bioavailability and interactions and the appearance of immune-stimulants (Lim & Webster, 2001).

Another issue in the aquaculture industry is the demand and supply in the market. As argued by Barman *et al.* (2013) and Lovell (1989) the affected invisible force of the market that results in an economic disadvantage to the industry are the persistent infectious diseases affecting the fishes. Hence, studies diverted its attention to the method of reducing these infectious diseases. There was the introduction of antibiotics to combat bacteria that was used to infect the fish. While looking for an antibiotic method to combat bacteria, another issue arose. The farmers had an economic loss due to poor knowledge of administering the available antibiotic nutritional products. Also, this method is treatment effect, and as the sick fish do not often eat the medicated feed during illness, it was difficult to establish the appropriate dosage of these antibiotics (Anderson, 1992). Due to the large disadvantages of this method to fish health, Ogier de Baulny *et al.* (1996) suggested a preventive method. This paved the way for a vaccine to emerge as a means of preventing bacterial infected diseases. Nevertheless, this did not bring a permanent solution. There are other notable diseases in aquaculture industry that could cause low output in the aquaculture industry (Raa *et al.*, 1992). As an alternative, introducing immunostimulants by modification of the fish nutritional diet would prevent and resist bacterial infected diseases (Sakai, 1999; Jadhav *et al.*, 2006).

2.10.1 Immunostimulants in nutritional fish diets

This method of immunostimulants emerged due to increase response of the fish against any pathogenic bacteria. It is a supplement and non-nutritive (Lim & Webster, 2001; Jadhav *et al*., 2006).The recent argument is that the immunostimulants introduced plays significant role working out the health system of the fish (Barman *et al.*, 2013). This is because fish, unlike mammals, does not depend on certain defensive mechanism rather

on broad-spectrum protection mechanism (Lovell, 1989; Barman *et al.*, 2013). As pointed out in Dautremepuits *et al.* (2004); Kawakami *et al*. (1998) and Villamil *et al*. (2003), the efficient products for immunostimulant for fish feeding include chitin, levamisole, vitamins, peptidoglycan, Beta-glucans (β-Glucan), chitosan and yeast. Microalgae, microorganisms, polysaccharides herbal extracts that are cost-effective, environmentally friendly with no health threatening consequence on faunae and humans are currently used in aquaculture industry (Sakai, 1999; Kim *et al.*, 2002; Harikrishnan, *et al.*, 2003; Li & Gatlin, 2005; Supamattaya *et al.*, 2005; Salnur *et al.*, 2009; Kumar *et al.*, 2011; Şahan *et al.*, 2016). They also have antioxidant, anti-stressors and antimicrobial agents which may reduce the cost of managing diseases by averting the expenses attained from using antibiotics, chemicals, and vaccination (Harikrishnan & Balasundaram, 2005).

Previous experimental work of Dalmo *et al.* (1996) and Mulero *et al.* (1998), suggested that feed fish diet need to have inclusion level of 50 - 200µg/ml β-Glucan. This increases the survival rate of Atlantic salmon against *Vibrio salmonicida* and *Vibrio anguillarum*. Gilthead sea bream Fed with levamisole does increase the lymph Okine, phagocytosis, complement activity and respiratory burst production (Mulero *et al.*, 1998). A similar study on using fish feed with levamisole while testing for rainbow trout and carp showed that there is a modulator to enhance non-specific immune activity and resistance towards pathogenic bacteria in fish health and growth (Siwicki, 1990; Gannam & Schrock, 1999; Findlay *et al.*, 2000).

Spirulina and *Chlorella* have received much attention as immunostimulants owing to their *in vitro* and *in vivo* immunostimulating effects (Miranda *et al.*, 1998). *Spirulina* was found to provide immunity to *Labeo rohita* fingerlings and juveniles *Olive flounder* after *A. hydrophila* and *Edwardsiella tarda* infections respectively (Andrews, *et al.*, 2011; Kim *et al.*, 2015). On the other hand, *Chlorella* has been found to improve

survival and immunity of larval of Korean rockfish and gibel carp (Cho *et al.*, 2001; Xu *et al.*, 2014) and no negative consequence on liver functions of rohu after *Aeromonas hydrophila* challenge (Pradhan & Das, 2015). It can as well be involved in regulating adaptive and instinctive immunity (Zhang *et al.*, 2014).

2.10.2 African catfish and health maintenance

To maintain fish health, understanding some diseases will be required. In the tropical regions, African catfish is often susceptible to common diseases and pathogens such as *Dactylgyrus*, *Chilodonella*, and *Costia* found in a pond (Viveen *et al.*, 1986). This was due to metazoan and protozoan infections. To control the spread of these diseases, it was suggested by Viveen *et al.* (1986) that treatment method with organic phosphate esters that include *Masoten*, *Bromex* and *Dipterex* should be applied. On the other hand, during hatchery of African catfish, it was recommended that an antibiotic should be added to the fish feed. Oxytetracycline and chloramphenicol are the common antibiotics available for the formulation of the fish feed. This is because the fish infections have a strong connection with changes in the environment like the quality of water in the pond, the temperature and the farmers' carefulness in handling the fish.

Boon *et al.* (1987) reported that the African catfish fingerlings are susceptible to ruptured intestine syndrome (RIS) which refers to open belly disease (OBD). This disease often occurred during five to eight weeks' post-hatch, when the fish is exposed to an elevated level of feeding. The death rate of the fingerlings of the African catfish becomes high because the caudal part of the intestine would be ruptured as caused by the disease. In addition, the broken head disease is commonly observed in brood fish kept at high-density tank characterised by an inflammation of fish skull because of lateral skull break (Huisman & Richter, 1987). Hence, in such an environment where there is a concentration

of waste from fish feed because of the fish low appetite can leading to an epidemic of the disease (Huisman & Richter, 1987).

2.10.3 Modulatory roles of dietary immunostimulants against pathogenicity of *A. hydrophila*

Diseases such as scale protrusion disease, haemorrhagic septicaemia, dermal ulceration and fin rot disease are associated with *Aeromonas hydrophila*, which arises from pathologic conditions, are common in aquaculture farming (Cipriano *et al.*, 1984). The type of organism that usually causes infectious disease through freshwater, brackish and warm-water fish species is the motile aeromonad species (MAS). The MAS bacteria types are *Aeromonas caviae, Aeromonas hydrophila* (gram-negative) and *Aeromonas sobria.* These bacteria usually occur as an opportunist. The bacteria spread because the fish is exposed to stress during mobility, skin injury, xenobiotic condition and environmental stressor like an unusual change in temperature (Lim & Webster, 2001).

Further, previous studies demonstrated that the immune system of a fish has sufficient findings attributable to immunostimulants feed formulated supplement from animal and plant extracts. For example, chitosan diet intake was found to be more effective for fish survival or immune effective than chitin and levamisole in common carp challenged with *Aeromonas hydrophila* (Gopalakannan & Venkatesan, 2006). In a more recent study by Lin *et al.* (2009), the application diet of chitosan- oligosaccharides supplement was found to be very effective in rainbow trout. According to Verma *et al*. (2013), *Aeromonas hydrophila* that usually infects African catfish, was investigated with plant immunostimulants supplement with medicinal characteristics. It was observed that supplementing African catfish with *Ficus benghalensis* and *Leucaena leucocephala* provided antibacterial properties. When the fish is faced with serious metal pollution, El-Boshy *et al.* (2014) suggested that adding fucoidan in the diets formulated feed of African

catfish increase cellular and humoral immunity in the African catfish. It equally increases the capability to develop pathogenic bacteria resistance. Supplementing soybean with iron was found to maintain normal immunity for the resistance of *Edwardsiella ictaluri* bacteria in Channel catfish (Barros *et al.*, 2002). The work of Davis and Hayasaka (1984) indicated that marine tunicate (*Ecteinascida turbinate*) extracted from invertebrate animal increases survival rates and phagocytosis level of eel after *Aeromonas hydrophila* injection.

In developing disease resistance for fish against infectious diseases, Sahu *et al.* (2007) evaluated garlic dosage required for a fish to put an elevated level of resistance. It was observed from the experiment that the mortality rate was reduced after 60 days. While survival decreased in the control group, the garlic treatment group increased. It was asserted that *Labeo rohita* fish developed higher resistant to infection in opportunist *Aeromonas hydrophila* when garlic, *Allium sativa* was introduced into the fish diet (Sahu *et al.*, 2007)

Supplementing the diet of African fish with 5% *Spirulina* increases the lysosome activity (Promya & Chitmanat, 2011) while Sayed and Fawzy (2014) reported an increase in haematological parameters when Fed with 5 $g^{-}k$ of *Spirulina*. To date, and to the best of the knowledge of the researcher, the effect of dietary *Chlorella* on disease resistance and immune stimulating capacity of African catfish is limited in the literature. Therefore, this thesis researched into the application of both *Spirulina* and *Chlorella* on the immunity of this commercially important freshwater fish.

2.11 Fish stress management, oxidative stress biomarkers and antioxidant activity.

It is no doubt that all living organisms are vulnerable to varieties of stress subject to environmental conditions. With stress in environmental conditions, reactive oxygen

species develop (Livingstone, 2001; Abele & Puntarulo, 2004; Valavanidis *et al.*, 2006; Bowden, 2008; Lushchak, 2011). Overproduction of ROS indicates oxidative stress (OS). OS and inflammation are both related to disease (Sayeed *et al.*, 2003; Amin & Hashem, 2012). This does affect the respiratory system of the organism as applied to African catfish. Uncontrolled exposure to stressful environmental condition increases the ROS-mediated oxidative damage (Stoliar & Lushchak, 2012). It equally influences oxidative stress. An increase in ROS affects cellular metabolism, regulation and even damage the components of the cell (Lushchak, 2011). In addition, Priyadarshani *et al.* (2012) emphasized that there is a need for redox balance. The redox balance is a delicate balance arising from the forces of pro and anti-oxidants. Basically, the pressure of antioxidants to prevent pro-oxidant of the cells brought about the oxidative stress (Priyadarshani *et al.*, 2012). Findings have shown that in fish farming the oxidative stress is common during deficiency in nutrition, abnormal temperature, xenobiotic exposure, and hypoxia (Avanzo *et al*., 2002; Kolkovski *et al*., 2000; Hwang and Lin, 2002; Dandapat *et al*., 1999). To reduce the hypoxia and temperature effect in aquaculture farming, Hwang and Lin (2002) and Kolkovski *et al.* (2000) suggested that use of vitamin C to control the resultant oxidative stress.

The occurrence of the oxidative stress, as argued by Stoliar & Lushchak (2012), causes an antioxidant response. The response enhances encoding the genes of the antioxidant enzymes and increasing the ROS scavengers. Though, as found in Halliwell and Gutteridge (2015) and Livingstone (2001), sometimes ROS expands beyond the normal level of depletion resulting in oxidative stress. Starvation had been observed to be responsible for the increase (Domenicali *et al.*, 2001). This is due to unfamiliar feed resulting in feed deprivation in animals. To manage this stress, the ROS, several studies pointed to glutathione S-transferase, superoxide dismutase and catalase and alkaline phosphatase (ALP) as effective antioxidant enzymes pertinent to fish immune capability

(Sun, 1990; Fırat *et al.*, 2011; Amin & Hashem, 2012; Leaver *et al*., 1993; Fisher & Burggren, 2007).

2.11.1 Catalase

For example, Sun (1990) described the catalase enzyme as an element effective in catalysing the decomposition of hydrogen peroxide (H_2O_2). The enzyme produces water molecular oxygen that helps to protect the cell against damage caused by oxidative stress.Tiana *et al.* (1998) reported that catalase decreases mitochondria of rat liver as age increases. Though there are limited studies of catalase in fish production, Ogunji *et al.* (2011) and Rueda-Jasso *et al.* (2004) argued that catalase activities increased in carp Fed with maggot meal. The meal contains high lipid content that resulted in rapid growth rate and good feeding of the carp. However, moderation of catalase activities was observed in starved dentex liver (Morales *et al.*, 2004). No effect was found in rainbow trout (Hidalgo *et al.*, 2002). Pascual *et al.* (2003) reported that catalase decreases in sea bream with partial food restriction and fasting fish.

2.11.2 Superoxide dismutase

Taking the superoxide dismutase (SOD) for antioxidant enzyme, Winston and Di Giulio (1991) argued that superoxide dismutase is one of the effective antioxidants in the animal. Like in the case of catalase, superoxide dismutase activities in ageing rat liver have a decreasing effect (Tiana *et al.*, 1998). Also, Camougrand and Rigoulet (2001) positioned that increasing the consumption of mitochondrial oxygen improves production of O_2^-. As such, the superoxide dismutase does convert the superoxide anion (O_2^-) into less harmful H_2O_2 suggesting that the process was catalysed by catalase and glutathione peroxidase into oxygen and water.

Studies have shown that the presence of copper in the fish diet stimulates antioxidant enzymes including CAT and SOD. For example, the findings of Guillaume (2001) indicated that copper inadequacy in fish diet usually causes a decline in superoxide dismutase and cytochrome oxidase activity. This will influence cataract formation in fish. In the experimental work of Shao *et al.* (2014), they identified higher levels of catalase and superoxide dismutase in black sea bream that were Fed with protein concentrate and soya bean formulated diet than the control diet. The study of Han *et al.* (2011) showed that the feed containing selenium supplement in feeding gibel carp decreases the quantity of serum superoxide dismutase.

2.11.3 Glutathione S-transferase

In the case of glutathione S-transferase, it has detoxification properties. Studies have demonstrated that glutathione S-transferase has the capacity to detoxify endogenous toxic metabolites contamination arising from the environment together with tri-peptide glutathione GSH. Furthermore, it also carries out phase II lipid peroxidase detoxification (Nimmo, 1987; Leaver *et al.*, 1993) Also, Fisher and Burggren (2007) argued that glutathione S- transferase helps to detoxify toxins arising from pesticides, oil, and other hydrocarbons, as well as fortifies living creatures against peroxidative damage.

In some pollution studies, fish had been frequently used for monitoring the water contamination and biomarker of water because they are sensitive to the contaminated environment (Amado *et al.*, 2006a; 2006b). For example, Ogunji *et al.* (2007) observed that application of maggot meal in carp and tilapia did not have any significant effect on Glutathione S-transferase activity compared with fish Fed with fishmeal. The implication is that maggot meal does not have compound content capable of stimulating the enhancement of ROS. Abdelkhalek *et al.* (2015) reported that *Spirulina* supplementation improved altered biochemical parameters, tissue peroxidation, and antioxidant

biomarkers thereby decreasing deltamethrin induced toxic effects on *O. niloticus*. This according to the authors, is due to the radical scavenging and potent antioxidant activities of *Spirulina*. Similarly, *Spirulina* was found to increase catalase, the activity of glutathione reductase as well as reduce malondialdehyde (MDA) formation thereby increasing the antioxidant protective capacity of mono-sex tilapia (*Oreochromis niloticus).*

In another study, *Chlorella*, *Spirulina* and *Azolla* at 50% supplementation has been found to increase vitamin C and E and decreases enzymatic antioxidants mainly SOD, CAT and LPx an indication that resulting feed from these algae are not toxic and stressful to *Macrobrachium rosenbergii* postlarvae (Radhakrishnan *et al.*, 2014). In this respect, finding out the substance that could be harmful to African catfish feed is important. Therefore, there is the need for a nutritional study to determine GST activities in fish as a biomarker of oxidative stress in fish under culture condition.

2.11.4 African catfish and oxidative stress

In managing oxidative stress in catfish, quercetin additive had been used to improve antioxidant and prevent peroxidation in silver catfish fish (Pês *et al.*, 2016). In dealing with environmental copper toxicity, Abdel-Tawwab *et al*. (2007) suggested that adding organic selenium with 0.3g per kg would increase hepatic glutathione peroxidase. This will protect the cell membranes against oxidative damage in African catfish (Abdel-Tawwab *et al.*, 2007). The recent study of Ibrahim and Harabawy (2014) on carbofuran in *Clarias gariepinus* indicated that exposure to carbofuran brought a significant reduction in antioxidant enzymes such as Glutathione S-transferase, catalase, superoxide dismutase and Glutathione peroxidase in African catfish organ. Taufek *et al.* (2016) reported an increase in CAT antioxidant activity in African catfish when fishmeal was completely replaced by cricket meal.

2.12 Research gap

From the reviewed previous studies, there have been very few studies where palatability and digestibility of diets containing dried microalgae powder have been evaluated in fish (Burr *et al*., 2011). Although, various replacement studies have examined *Spirulina* and *Chlorella* for freshwater teleost (omnivorous and carnivorous) diets (Badwy *et al*., 2008; Abdel-Tawwab & Ahmad, 2009; Abdulrahman & Ameen, 2014; Xu *et al*., 2014; Kim *et al*., 2015; Pradhan & Das, 2015; Sarker *et al*., 2016; Khani *et al*. 2017), and recently *Spirulina* on catfish (Promya & Chitmanat 2011; Sayed *et al*., 2017). These studies mainly reported their effects on growth parameters and health with different results of inclusion levels in the fish feed of these parameters. Growth parameters and health are next to nutrient digestibility and are also influenced by several factors. Therefore, these parameters are not a direct indication of the actual digestibility of the nutrients in both algae. To estimate the efficacy of both *Spirulina* and *Chlorella* as possible protein substitutes in fish feed, there is the need for data on their nutrient digestibility. In comparison to growth studies, few studies reported the digestibility of diets containing these two freshwater algae. From these previous studies, fewer reported the digestibility of amino and fatty acids of the diets. The available data are limited to four fish species. Apparent digestibility coefficient of *Spirulina* in Caspian great sturgeon, Arctic charr and Atlantic salmon (Burr *et al*., 2011; Safari *et al*., 2016) and *Spirulina*, *Chlorella* spp and *Schizochytrium* spp (Sarker *et al*., 2016) in Nile tilapia. Recently, Teuling *et al*. (2017) compared the effect of cell wall characteristics on the nutrient utilisation of four different algae spp namely *Arthrospira maxima, Chlorella Vulgaris, Scenedesmus dimorphus* and *Nannochloropsis gaditana* on Nile tilapia and African catfish. However, the work omitted data on the digestibility and availability of fatty acids and amino acids components of both algae as relevant to this commercially important freshwater fish.

Considering the importance of digestibility of the amino acids and fatty acids, this current study filled the gap because SP and CL are the freshwater microalgae species often used in fish feed mainly as nutritional supplements or in combination with other sources of protein. Besides, this digestibility data would carefully guide in the addition of these microalgae into fish diets. Hence, this study examined the *C. gariepinus* growth response in addition to the apparent digestibility coefficient of macronutrients, amino acids and fatty acids in both *Spirulina* and *Chlorella* in relation to fishmeal (reference diet).

After filling the gap of digestibility, the need arises to determine the growth performance of the catfish feeding trials and the proximate body composition of the experimental fish. There are several kinds of literature that discussed growth performance with mixed results.

In consequence of the characteristics of both algae, does replacement of fishmeal with *Spirulina* and *Chlorella* influences the African catfish production in terms of growth and body composition? To this effect, this study experimentally investigated the dietary effects of graded levels of *Spirulina* (SP) and *Chlorella* (CL) as fishmeal (FM) protein replacement on the growth performance, body composition and survival rates of African catfish *C. gariepinus.*

African catfish is regarded as one of the most important freshwater fish due to its high growth, reproduction and adaptability rate in Africa, Southeast Asia and other parts of the world (Fasakin *et al.*, 2003; Al-Dohail *et al.*, 2009). It has superior growth performance compared with other *Clarias* species, hence there is a need for a well-balanced diet to sustain and increase the commercial production of this important fish species at a reasonable cost to both farmers and consumers. Promya and Chitmanat (2011) recommended 50 g kg^{-1} dietary inclusion of Cladophora + basal diet and *Spirulina* + basal diet to increase growth performance as well as carotenoid and immunity of *C. gariepinus,*

respectively. Inconsistent results were found in the literature regarding the optimum dietary addition levels of *Spirulina* and *Chlorella* for growth performance of most fish species. Also, there is a dearth of information on the effect of *Chlorella* on *C. gariepinus*.

Based on the excellent nutritional and health properties of the two algae as discussed previously in this thesis, their usefulness to growth and body composition of the fish has attracted much research attention. However, few studies compared the effect of either *Spirulina* or *Chlorella* or either of the two algae with other microalgae as a substitute for fishmeal in other fish species and crustaceans. Some of the studies compared *Spirulina platensis, Chlorella vulgaris* and *Azolla pinnata* on *Macrobrachium rosenbergii* (Radhakrishnan *et al.*, 2014), *Chlorella* and *Scenedesmus* (Badwy *et al.*, 2008), or *Chlorella* and *Spirulina* with *Schizochytrium* sp. on Nile tilapia (Sarker *et al.*, 2016). Nevertheless, their findings ignored the optimal inclusion level to replace the fishmeal protein. To the best of the understanding from the literature search, comparing the effect of *Chlorella* and *Spirulina* on African catfish got little or no attention. This study, unlike the previous studies, experimentally compared the effect of *Spirulina* and *Chlorella* Separately in pelleted diets of *C. gariepinus* to ascertain the effects of altered inclusion levels on the growth enhancements and carcass composition. Additionally, the study determined the comparative advantages of one alga over the other, as well established the optimal level that positively replaced fishmeal without adverse effect on *C. gariepinus* growth.

Thirdly, the establishment of a perfect balance between growth and health conditions of fish has been proffered as a way of achieving sustainable aquaculture (Kaleeswaran *et al.*, 2012). Research has shown that the general health and immune responses of fish are strongly related to their nutrition (Priya *et al.*, 2004; Kumari & Sahoo, 2005), hence, the use of dietetic immunostimulants instead of antibiotics in the

prevention and control of fish diseases and in dealing with unsuitable environmental conditions is of increasing interest in aquaculture research.

Prevalence of the zoonotic diseases has also caused significant economic losses to farmers which might likely to be due to poor sanitation and feeding issues. Farmed fish are susceptible to numerous diseases which may be bacterial, viral, and fungal or of other parasites in origin (Camus, 2004; Toranzo *et al.*, 2005). For example, *Aeromonas hydrophila* is a widely distributed pathogenic gram-negative bacterium (Sabur, 2006), which has been linked with the epizootic ulcerative condition and other infections in countries like Malaysia, Sri Lanka and Japan (Miyazaki *et al.*, 2001; Mathur *et al.*, 2005; Samal *et al.*, 2014). *Aeromonas* species such as *A. sobria* and *A. caviae* have been linked with most harmful infectious diseases afflicting African catfish (Janda & Abbott, 2010). Symptoms of infection with this bacterium include skin lesions and haemorrhagic Septicaemia (Law, 2001; Andrews *et al.*, 2011; Ahamad *et al.*, 2013; Anyanwu *et al.*, 2015). Also, distended abdomen, red mouth, and haemorrhages surrounding the anus of fish (Alain, 2009). The mortality rate is usually high, resulting in huge financial losses to fish farmers globally. So, therefore, there is the need to produce feed, not only as growth promoters but as immunostimulants for preventing bacteria and opportunistic pathogens from proliferating, to avoid antibiotic use which may have a negative impact on the cultured fish due to the accumulation of antibiotic residues (Schmidt *et al.*, 2000).

Research has shown that *Spirulina* and *Chlorella* had received much attention as immunostimulants owing to their *in vitro* and *in vivo* immune-stimulatory effects (Miranda *et al*., 1988). It has been shown to possess immune-stimulatory and immunity-enhancing properties, improving growth and survival, enhancing pigmentation and digestion, and having scavenging and peroxidation properties (Spolaore *et al.*, 2006; Gupta *et al.*, 2007; Zhang *et al.*, 2014). In Malaysia, Nigeria and other parts of the world,

catfish especially *Clarias gariepinus*, is a commercially important valuable fish species which has gained prominence in aquaculture. It also has wide acceptability among consumers (Yisa & Olufeagba, 2005; Akinrotimi *et al.*, 2014). The physiology of catfish, particularly the effect of *Spirulina* on its growth, haematology and immunity has been well studied in recent. However, to the best of the researcher's knowledge, the effect of *Chlorella* on the nutrition and immunity of *C. gariepinus* has not received attention in the literature. Therefore, considering the nutritive and immunostimulatory properties of both *Spirulina* and *Chlorella*, the present study evaluated and compared the effect of dietary *Spirulina platensis* (*Arthrospira platensis*) and *Chlorella vulgaris* on growth, blood parameters, and the immune status of the *C. gariepinus* challenged with *A. hydrophila.* Results from this study could provide the basis for the use of *Chlorella* as a replacement for fishmeal protein in *C. gariepinus*. Additionally, the findings would provide additional information to the existing literature regarding the dietary effect of *Spirulina* on this economically important freshwater fish species.

Fourthly, to combat free-radical damage, terrestrial and aquatic animals utilize many defense mechanisms, which include antioxidant enzymes, such as glutathione S-transferase (GST) catalase (CAT), and superoxide dismutase (SOD), and compounds like glutathione, ascorbic acid, polyphenolics, carotenoids and a-tocopherol (Kruger & Mann, 2003; Ahmed *et al.*, 2017). Although the antioxidative defence can be reduced by an increase in the level of pollutants, thereby lowering fish production, this problem could be practically solved by lessening oxidative stress, as well as consequent damage via dietary supplementation using natural nutraceuticals and antioxidants (Jayakumar *et al.*, 2011).

S. platensis is a freshwater microalga with abundant fatty acids (gamma-linolenic acid (GLA), vitamins, antioxidant pigments like carotenoids, essential amino acids,

protein, and minerals (Augustin *et al.*, 2011). Several authors have used dried *S. platensis* as a feed supplement (Augustin *et al.*, 2011; Kim *et al.*, 2013; Radhakrishnan *et al.*, 2014), and explored as a partial replacement. For instance, in *Peneaus semisulcatus* feed (Ghaeni *et al.*, 2011), Pacific white shrimp *Litopenaeus vannamei* feed (Hanel *et al.*, 2007), Guppy fish feed (Dernekbasi *et al.*, 2010) on growth and feed conversion in guppy and white shrimp *Litopenaeus schmitti* feed (Jaime-Ceballos *et al.*, 2005). Supercritical fluid obtained from *S. platensis* exhibits antimicrobial and antioxidant activity (Mendiola *et al.*, 2007). It is safe and employed as a substitute for commercially available synthetic antioxidants.

C. vulgaris is a freshwater based single-celled algae, which contains the highest quantity of chlorophyll of all plants. It is a superfood with an abundant nutrient containing various vitamins and minerals, 18 amino acids (with the entire essential amino acids inclusive), and 60% protein. *Chlorella* Growth Factor (CGF), which is a phytonutrient is one of its unique properties. CGF is abundant in the nuclei of algae, made up of vitamins, nucleic acid associated substances, amino acids, proteins, peptides and sugars of specific interest because of purification with the aid of peptide glutathione in the CGF (Nick, 2003). Over 20 minerals and vitamins are present in *C. vulgaris*. These include iron, potassium, calcium, phosphorous, magnesium, pro-vitamin A, vitamins B1, B2, B2, B5, B6, B12, C, E and K, biotin, folic acid, inositol, plus vitamins C, E and K. *C. vulgaris* exhibits a better activity towards inhibiting peroxidation of lipid when compared with glutathione and exhibit antioxidant properties (Bengwayan *et al.*, 2010).

Hence, in view of the antioxidant and nutraceutical properties of both SP and CL, and the fact that little has been reported in literature about the relation between *Spirulina* and *Chlorella* and antioxidant defences in catfish, this research explored the effect of *S. platensis* and *C. vulgaris* supplementation as a partial replacement for fishmeal on

parameters such as growth, haemato-biochemical and antioxidant response in African catfish.

2.13 Conceptual framework

Having reviewed the various literature on digestibility, growth, immunology and anti-oxidation, this thesis presents the conceptual framework in Figure 2.2.

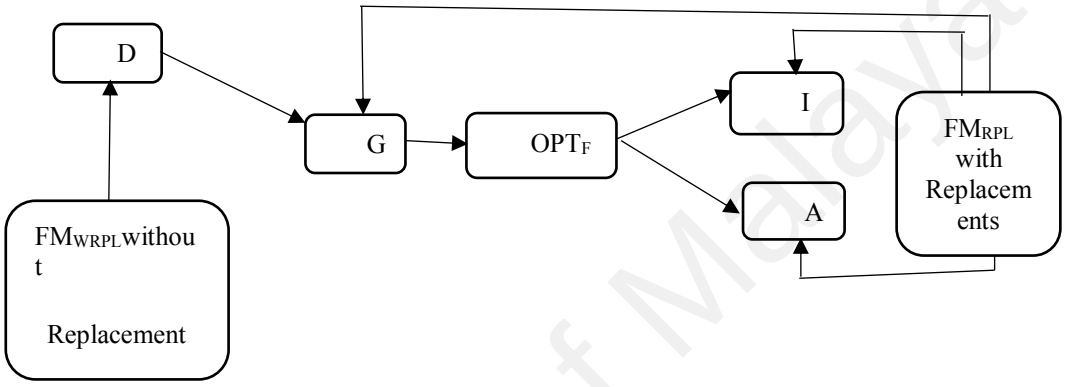


Figure 2.2: The conceptual framework

Note: DGB represents Digestibility; GP represents growth performance; FMwrpl represents feed formulation without replacement; OPT_{FMRL} represents optimum fishmeal replacement levels with *Spirulina* or *Chlorella*; IMS represents immunology S.; AOS represents anti-oxidative Stress; FMRPL represents fishmeal formulation with replacement.

The above framework accounts for the processes in achieving the objectives as discussed in the literature. Firstly, understanding appropriate fish feed, the fish digestibility was determined through preparing a feed formulation at the ratio of 70: 30 of reference: test diets (FM, SP and CL) without replacement of FM and applied on the African catfish. Secondly, the growth performance of the fish was determined when Fed with feed whose fishmeal was replaced with either SP or CL at 12.5%, 25%, 50% and

75%. Thirdly, the growth performance determination enables us to proceed to understand the levels of FM replacement with either SP or CL at which the optimum growth was achieved. Fourthly, understanding the optimum growth replacement levels, the paths of understanding the immune system of the fish and the extent to which the fish can resist oxidation stress with the fish feed containing *Spirulina* and *Chlorella* was opened. After that, the efficiency of the *Spirulina* and *Chlorella* meals on the African catfish was known and the conclusion was drawn.

CHAPTER 3: METHODOLOGY

3.1 Introduction

This chapter deals with the research processes undertaken to achieve the research objectives of this thesis. The major sections of the chapter include research design, materials and methods, experimental set setup and data analysis.

3.2 Research design

In scientific research, research design is a template for conducting a scientific study with a maximum control of the methods to enhance result reliability and trustworthiness (Barnes *et al.*, 2003). Hence, there are many research designs available in scientific researches, namely: descriptive research design, explorative research design, experimental research design and Quasi-research design (Walker, 2005). In this thesis, the study selected the experimental research design to achieve the four objectives. The experimental research design was selected because it helps to determine the cause-effect of the dependent and independent variables (Walker, 2005). So, the flowchart of the experimental procedure is shown in Figure 3.1.

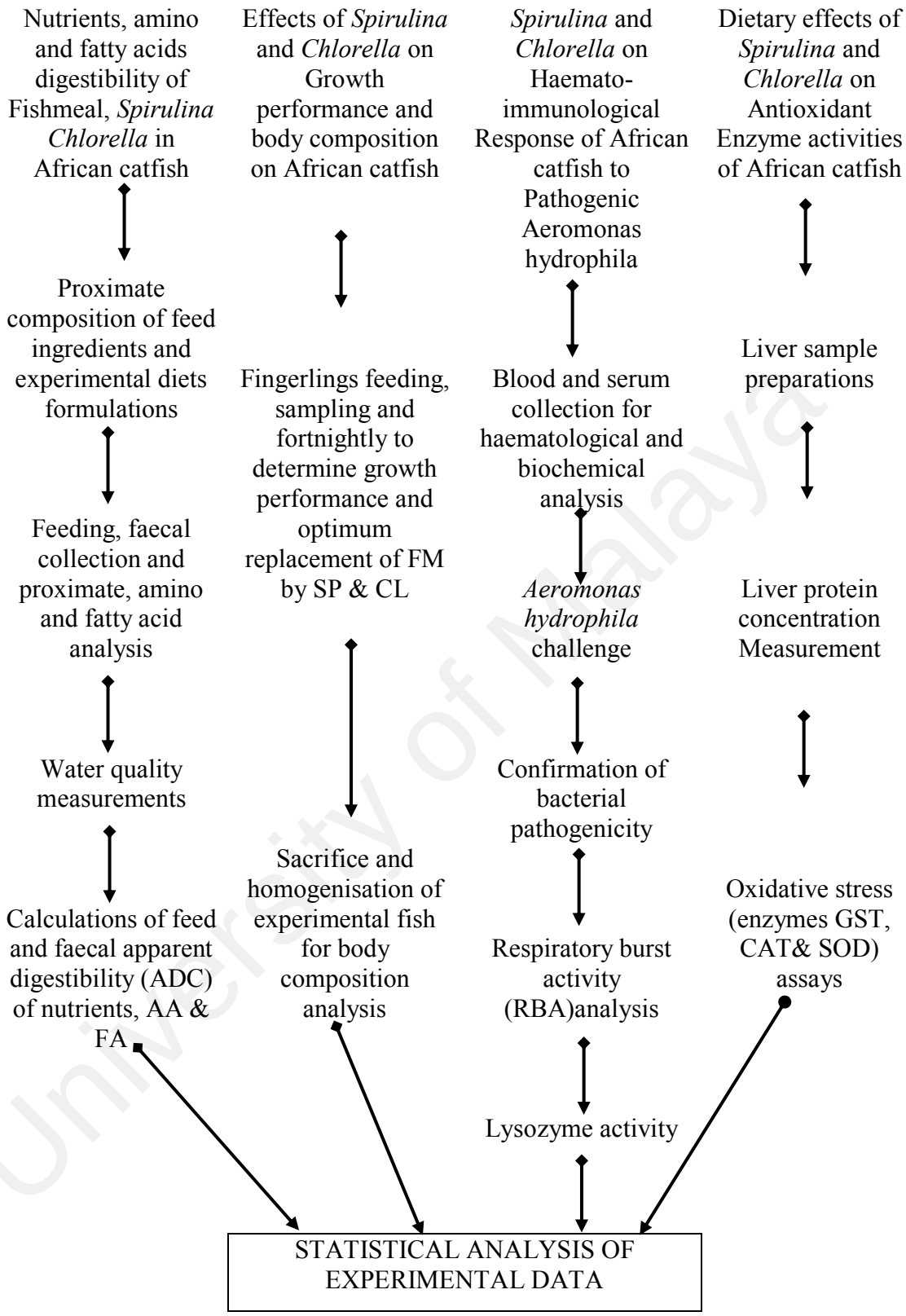


Figure 3.1: Flowchart of the experimentation procedure

3.3 Experimental research

According to the experts in pure sciences and medicine, experimental research is the type of research that "provides the framework for establishing a relationship between cause and effect" (Walker, 2005; Mulhall, 1994). Another argument for using experimental research is that the researcher uses the deductive method because he is an active agent in controlling the cause (independent variable) and observes the effect (dependent variable) (Walker, 2005; Polit *et al*., 2006; Proctor, 1998; Newell, 1994).

3.4 Materials and Methods

This section focuses on the material and methods used to arrive at each objective's outcome. It contains all the experimental procedures adopted in the thesis which follows the structure of experimental research design (Bernard *et al.*, 2016). Although the study could not divide the section according to the objectives of the thesis to avoid repeating the methods, this section is divided into subsections that explain all the experimental research procedures starting from digestibility methodological procedure and collection of feed.

3.4.1 Experimental diets and formulations

Spray dried *Spirulina* and *Chlorella* (thin cell and cell-ruptured) powder used for these studies were purchased from TST Bioceuticals (Perak, Malaysia). Prior to starting the experimental studies, proximate, amino and fatty acid compositions of both microalgae and other feed ingredients (Appendix A, B and C) were carried out before they were used to formulate experimental diets based on the sizes and protein requirement of the different groups of catfishes used in the different studies in this thesis.

For digestibility diets (Table 3.1), a high-quality reference diet and combined with each test microalgae species (*Spirulina* and *Chlorella*) at a 7:3 percentage to get two test

diets were formulated. The test diets were formulated using 30% of each of the test ingredients (*Spirulina* and *Chlorella*) and 70% reference diet on a dry basis as described in the apparent digestibility methodology of Bureau and Hua (2006) and Cho and Slinger (1979). The chromic oxide of 1% was applied as an inert indicator. Tables 3.2 and 3.3 show the amino and fatty acid compositions of the diets

Table 3.1: Gross (Kcal 100 g^{-1}) and chemical (g 100 g^{-1}) composition of the experimental diets Fed to *C. gariepinus* juveniles.

	Experimental Diet		
Ingredients	**REFDT**	**SPDT**	**CLDT**
Fishmeal	300	0	0
Spirulina	0	300	0
Chlorella	0	0	300
Soybean	277.30	277.30	277.30
Rice Bran	87.10	87.10	87.10
Corn Meal	237.10	237.10	237.10
Fish+ Palm oil	285	285	285
a Premix Vitamin	15.00	15.00	15.00
b Premix Mineral	15.00	15.00	15.00
Lysine	10.00	10.00	10.00
Methionine	10.00	10.00	10.00
Chromium Oxide	10.00	10.00	10.00
Di-clorophosphate	10.00	10.00	10.00
Total	1000	1000	1000
Proximate composition			
Moisture	8.35±0.01	7.87±0.02	7.48±0.01
Crude Protein	39.75±0.02	41.00±0.22	39.38±0.02
Crude Lipid	8.81±0.02	8.04±0.01	8.51±0.01
Crude Fibre	0.8±0.06	0.97±0.01	1.21±0.01
Crude Ash	8.69±0.01	8.50±0.02	8.63±0.01
Nitrogen Free Extract	33.60±0.04	33.62±0.25	34.79±0.03
Gross Energy Kcal 100 g-1	442.24±0.31	442.11±0.31	442.08±0.12

[a] Vitamin premix supplied: vitamins A,500IU; B1,1.0mg; B2,0.5mg; B3,0.3mg; B6, 0.2 mg; B12,0.001 mg; C,0.1 mg D3 100IU; E,0.75 mg, K,0.02 mg; niacin,0.2 mg, folic acid,0.1 mg; biotin,0.24 mg; pantothenic acid,1.0 mg; inositol, 2.5 mg. [b] Mineral premix provided the followings per kg diet: iron, 8.0mg; selenium, 0.2mg; magnesium oxide, 0.6 mg; manganese, 1.0 mg; zinc, 8.0 mg; copper, 0.15mg; potassium chloride, 0.4 mg; sodium bicarbonate, 1.5 mg; iodine,1.0 mg; cobalt, 0.25 mg. REFDT: Reference diet; SPDT: *Spirulina* diet; CDT: *Chlorella* diet.

Table 3.2: Amino acid profile of the experimental diets Fed to *C. gariepinus* juveniles

Amino acids	REFDT	SDT	CDT	P value
Hydroproxiline	0.09±0.00 [a]	0.09±0.01[a]	0.08±0.00 [a]	.317
Aspartic acid	2.63±0.00 [b]	2.74±0.01 [a]	2.16±0.00 [c]	.000
Serine	1.54±0.01[b]	1.63±0.01 [a]	1.29±0.01 [c]	.000
Glutamic acid	4.89±0.01[b]	4.96±0.02 [a]	3.91±0.01[c]	.000
Glycine	1.10±0.01[c]	1.46±0.02 [a]	1.24±0.01 [a]	.000
Histidine*	0.72±0.01 [c]	0.84±0.02 [b]	0.95±0.02 [a]	.000
Arginine*	2.03±0.01[c]	2.40±0.01[a]	2.36±0.01[b]	.000
Threonine*	2.05±0.01 [c]	2.47±0.02 [a]	2.14±0.01 [b]	.000
Alanine	1.17±0.01 [c]	1.70±0.01 [a]	1.42±0.00 [b]	.000
Proline	1.34±0.01 [b]	1.51±0.01 [a]	1.35±0.01 [b]	.000
Tyrosine	0.64±0.01 [b]	0.82±0.01 [a]	0.46±0.01 [c]	.000
Valine*	1.32±0.01 [b]	1.73±0.02 [a]	1.74±0.01 [a]	.000
Methionine*	1.72±0.01 [b]	1.82±0.01 [a]	1.69±0.01 [b]	.000
Lysine*	2.55±0.01[c]	3.59±0.01 [a]	3.55±0.01[b]	.000
Isoleucine*	1.13±0.01 [c]	1.43±0.01 [a]	1.30±0.01 [b]	.000
Leucine*	2.91±0.02 [c]	3.55±0.01 [a]	3.45±0.02 [b]	.000
Phenylalanine*	1.54±0.01[b]	1.75±0.01 [a]	1.75±0.01 [a]	.000
Tryptophan*	0.45±0.01 [b]	0.41±0.01[c]	0.50±0.01 [a]	.001

Values are means of 3 replicates/treatments ± standard error (SE). Means with different superscript (a, b & c) within the same rows are statistically significant ($p < 0.05$) * Essential amino acids (EAA) REFDT, SDT &CDT: Reference, *Spirulina* and *Chlorella* diets.

Table 3.3: Fatty Acid Profile of the experimental diets Fed to *C. gariepinus* Juveniles

FA	REFDT	SDT	CDT	P Value
SFA	40.28±0.02[c]	42.78±0.02 [a]	41.30±0.02[b]	.000
MUFA	24.25±0.38 [a]	16.57±0.11 [b]	14.43±0.22 [c]	.000
PUFA	35.47±0.59 [c]	40.65±0.39 [b]	44.27±0.07 [a]	.000
C182n6t	0.47±0.01 [ab]	0.49±0.02 [a]	0.44±0.01[b]	.105
C182n6c	13.14±0.01 [c]	14.29±0.02[b]	31.57±0.02 [a]	.000
C183n4	0.69±0.02 [c]	3.41±0.01 [a]	0.81±0.01 [b]	.000
C183n6	0.44±0.01 [a]	0.43±0.01 [a]	0.45±0.01 [a]	0.51
C183n3	10.79±0.02 [c]	11.00±0.01 [b]	12.17±0.02[a]	.000
C184n3	6.72±0.01 [c]	7.50±0.01 [b]	8.13±0.01 [a]	.000
C203n6	0.55±0.01 [a]	0.46±0.02 [b]	0.43±0.01[b]	.002
C203n3	8.79±0.02[c]	9.10±0.03 [b]	10.20±0.12 [a]	.000
C205n3	7.55±0.02 [a]	6.56±0.02 [b]	6.59±0.02 [b]	.000
C226n3	7.33±0.01[c]	11.95±0.02 [a]	11.87±0.02 [b]	.000
N3/n6	2.82±0.01[b]	2.94 ±0.02[a]	1.49 ±0.03 [c]	.000

Mean values with different superscript across the rows (a-c) were significantly difference $p < 0.05$. N = 3 ± standard error (SE). FA: Fatty acids; TFA: Total fatty acids; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acid, EPA: eicosapentaenoic acid, DHA: Docosahexaenoic acid, REFDT: Reference diet; SDT &CDT: *Spirulina* and *Chlorella* diets

For growth performance study**,** nine diets (Table 3.4) were prepared in sets of either *Chlorella* (CL) or *Spirulina* (SP) to replace fishmeal at various inclusion levels of 0, 12.5, 25, 50 and 75 % resulting in diet levels of SP12.5 to SP75 % or CL12.5 to CL75 %. All feeds were iso-nitrogenous at 45% crude protein. Table 3.5 and 3.6 shows amino and fatty acid compositions of the experimental diets.

Table 3.4: Formulations and proximate composition of experimental diets g kg^{-1}

INGREDIENTS	TREATMENTS								
	Control	*Spirulina*				*Chlorella*			
	0.00%	**12.5%**	**25%**	**50%**	**75%**	**12.5%**	**25%**	**50%**	**75%**
Fishmeal	250	218.75	187.5	125	62.5	218.75	187.5	125	62.5
Spirulina	0	31.25	62.5	125	187.5	0	0	0	0
Chlorella	0	0	0	0	0	31.25	62.5	125	187.5
Soya bean	503	494.5	501.9	500.3	501.9	512.3	521.7	540.5	559.2
Rice bran	139.3	150.9	143.9	148.5	143.9	133.6	127.9	116.5	105.1
Vitamin premix a	4	4	4	4	4	4	4	4	4
Mineral premix b	5	5	5	5	5	5	5	5	5
Cod liver oil	59.7	56.6	56.2	53.2	56.2	56.1	52.4	45.0	37.7
Lysine	2	2	2	2	2	2	2	2	2
Methionine	7	7	7	7	7	7	7	7	7
DCP	10	10	10	10	10	10	10	10	10
Binder	20	20	20	20	20	20	20	20	20
Total	1000	1000	1000	1000	1000	1000	1000	1000	1000
Protein	46.21	46.36	45.86	45.78	45.67	45.78	45.45	45.32	45.12

Table 3.4, continued

INGREDIENTS	TREATMENTS								
	Control	*Spirulina*				*Chlorella*			
	0.00%	12.5%	25%	50%	75%	12.5%	25%	50%	75%
Fat	10.89	10.71	10.72	10.78	10.98	10.69	10.41	10.45	10.32
Ash,	10.16	10.28	10.09	10.14	10.18	10.47	10.45	10.41	10.53
Moisture	9.47	9.29	9.12	9.11	9.08	9.02	9.06	9.03	9.04
Crude fibre	2.56	2.78	2.67	2.78	2.86	2.51	2.56	2.69	2.71
NFE	20.71	20.58	21.54	21.41	21.23	21.53	22.07	22.10	22.28
Gross Energy	364.27	364.41	365.44	364.53	363.39	364.92	364.93	364.36	363.82

Vitamin premix supplied: vitamins A,500IU; B1,1.0mg; B2,0.5mg; B3,0.3mg; B6, 0.2mg; B12,0.001mg; C,0.1mg D3 100IU; E,0.75mg, K,0.02mg; niacin,0.2mg, folic acid,0.1mg; biotin,0.24mg; pantothenic acid,1.0mg; inositol, 2.5mg. Mineral premix provided the followings per kg diet: iron, 8.0mg; selenium, 0.2mg; magnesium oxide, 0.6 mg; manganese, 1.0 mg; zinc, 8.0 mg; copper, 0.15mg; potassium chloride, 0.4 mg; sodium bicarbonate, 1.5 mg; iodine,1.0 mg; cobalt, 0.25 mg

Table 3.5: Amino acids of the study diets containing graded levels of *Spirulina* and *Chlorella* g $100g^{-1}$.

Amino Acids	Control	SP12.5%	SP25%	SP50%	SP75%	CL12.5%	CL25%	CL50%	CL75%	**
HP	0.76±0.0	0.72±0.02	0.67±0.02	0.61±0.01	0.29±0.01	0.58±0.01	0.72±0.01	0.53±0.00	0.41±0.00	
Aspartic acid	7.23±0.16	7.15±0.09	9.04±0.19	7.28±0.01	6.07±0.05	8.85±0.02	5.19±0.00	8.01±0.01	6.62±0.01	
Serine	4.22±0.09	4.78±0.06	4.79±0.16	4.98±0.02	4.23±0.01	4.75±0.01	4.20±0.01	4.73±0.01	4.79±0.01	
Glutamic acid	13.37±0.35	13.03±0.13	16.28±0.36	13.86±0.01	11.32±0.01	15.91±0.00	9.96±0.02	14.39±0.01	12.11±0.04	
Glycine	4.18±0.11	4.34±0.05	4.25±0.08	4.58±0.00	3.71±0.01	4.37±0.00	3.99±0.01	4.10±0.01	4.08±0.01	
Histidine*	2.02±0.06c	2.07±0.02bc	2.11±0.06b	2.38±0.02a	1.85±0.00d	2.01±0.01c	2.17±0.00b	1.87±0.00d	2.00±0.00c	1.20
Arginine*	6.47±0.27b	6.57±0.06b	6.49±0.14b	7.47±0.09a	6.29±0.04c	6.10±0.01d	6.30±0.01c	5.76±0. 01e	6.08±0.04d	3.60
Threonine*	3.68±0.05c	3.78±0.05b	3.79±0.10b	4.43±0.06a	3.75±0.03b	3.82±0.02b	3.92±0.00ab	3.57±0.00d	3.77±0.01b	2.80
Alanine	3.69±0.04	3.60±0.04	4.30±0.13	3.90±0.06	3.50±0.02	4.34±0.01	2.84±0.01	4.17±0.00	3.68±0.03	
Proline	4.02±0.02	3.87±0.06	4.30±0.13	4.21±0.01	3.64±0.01	4.37±0.00	3.38±0.02	4.22±0.01	3.97±0.01	
Tyrosine	0.40±0.01	0.40±0.01	0.47±0.03	0.48±0.01	0.43±0.00	0.43±0.00	0.46±0.00	0.45±0.00	0.57±0.00	
Valine*	3.64±0.05b	3.70±0.05b	3.97±0.09a	4.14±0.04a	3.62±0.01b	4.09±0.20a	3.23±0.00c	3.62±0.00b	3.57±0.00b	2.40
Methionine a*	2.38±0.02d	2.40±0.04c	2.68±0.05b	2.91±0.02a	2.32±0.02d	2.64±0.01b	2.57±0.01c	2.21±0.00e	2.43±0.00c	2.30
Lysine*	5.40±0.14 c	5.09±0.08 d	6.12±0.14 a	4.87±0.09 d	4.67±0.02 e	6.12±0.08 a	4.64±0.01 e	5.71±0.01b	4.85±0.00 d	4.80
Isoleucine*	3.24±0.03 d	3.35±0.05 c	3.53±0.07 b	3.80±0.02 a	3.36±0.01 d	3.49±0.01 b	2.83±0.00 f	3.11±0.00 e	3.09±0.00 e	2.0
Leucine*	6.43±0.09 c	6.44±0.10 c	6.94±0.15b	7.18±0.04 a	6.28±0.02 cd	6.78±0.00 b	5.62±0.02 d	6.42±0.01 c	6.35±0.00 c	3.50
P/TAA*	3.87±0.07 d	4.04±0.06 c	4.07±0.09 c	4.80±0.00a	3.95±0.01 cd	3.97±0.01 cd	4.28±0.01 b	3.94±0.01 de	4.07±0.00 c	4.00
Tryptophan*	0.83±0.01a	0.79±0.00 a	0.79±0.00 a	0.78±0.00 a	0.70±0.00 b	0.76±0.00 b	0.72±0.01 b	0.68±0.00 bc	0.59±0.00 d	0.5

Values are means of triplicates of nine different feed samples. Mean values on the same row with different superscript (a - e) are significantly different ($P<0.05$); *: Essential Amino Acids ** Amino Acid requirement of *C. gariepinus*. Source: Uys (1989) and Unprasert (1994) a: Methionine + Cysteine
TAA: Phenylalanine /Total Amino Acid) HP: Hydroxyproline

Table 3.6: Fatty acid composition of diets containing various levels of *Spirulina* and *Chlorella* % of fatty acids.

Fatty acids	Control	SP12.5%	SP25%	SP50%	SP75%	CL12.5%	CL25%	CL50%	CL75%
TFA (mg/g)	42.62±0.01c	26.47±0.01e	48.81±0.01b	17.31±0.01 f	28.46±0.01d	52.48±0.01a	5.70±0.01h	13.39±0.01g	17.29±0.01f
SFA	46.08±0.01f	47.59±0.01e	47.83±0.01d	54.28±0.01 a	51.15±0.01b	47.91±0.01d	40.49±0.01g	50.11±0.01c	48.38±0.01cd
MUFA	23.05±0.01b	24.33±0.01a	23.16±0.01b	21.00±0.01d	20.12±0.01e	22.47±0.01c	15.46±0.01g	19.71±0.01e	18.94±.01f
PUFA	30.89±0.01c	28.12±0.01b	29.03±0.01g	24.73±0.02h	28.72±0.01g	29.64±0.01f	44.06±0.02 a	30.17±0.01d	32.70±0.01b
C4:0	0.07±0.01	0.14±0.01	0.00±0.00	0.73±0.01	0.00±0.00	0.27±0.01	0.96±0.01	0.00±0.00	0.00±0.00
C12:0	0.46±0.01	0.16±0.01	0.17±0.01	9±0.01	0.79±0.01	0.51±0.01	0.66±0.01	0.88±0.01	1.64±0.01
C13:0	1.52±0.01	0.95±±0.01	0.85±0.01	0.62±0.01	2.00±0.01	1.10±0.01	0.61±0.01	1.26±0.01	1.82±0.01
C14:0	2.42±0.01	2.46±0.01	2.05±0.01	2.60±0.01	1.82±0.01	2.38±0.01	2.14±0.01	2.67±0.01	4.84±0.02
C16:0	27.90±0.01d	31.77±0.02c	29.75±0.02d	35.90±0.02a	31.72±0.01c	30.63±0.01d	22.72±0.01f	32.43±0.02b	26.86±2.00de
C16:1	2.08±0.01	2.24±0.01	1.98±0.01	2.13±0.01	1.75±0.01	2.15±0.01	1.52±0.01	2.03±0.01	3.77±0.01
C18:0	9.50±0.01b	8.80±0.01d	8.89±0.01d	9.43±0.01b	10.25 ±0.01 a	9.28±0.01c	6.31±0.01 a	9.26±0.01c	2.39±0.0e
C18:1n9c	11.59±0.02 c	12.43±0.01a	12.61±0.01a	11.54±0.01c	11.82±0.01b	12.57±0.01a	7.38±0.01d	11.63±0.01c	2.52±0.0e
C18:1n7	1.56±0.01	1.61±0.01	1.21±0.01	1.62±0.01	1.23±0.01	1.11±0.01	1.08±0.01	1.46±0.01	2.20±0.01
C18:2n6t	0.23±0.01d	0.16±0.0 1e	0.21±0.01c	0.31±0.01b	0.25±0.01 d	0.38±0.01a	0.13±0.01e	0.21±0.01c	0.43±0.0a
C18:2n6c	13.00±0.01d	12.36±0.01c	13.60±0.01e	13.70±0.01f	15.00±0.01h	14.38±0.01g	9.51±0.01b	19.91±0.01i	5.43±0.0a
C18:3n4	0.37±0.01	0.44±0.01	0.83±0.01	1.17±0.01	2.05±0.01	0.59±0.01	0.22±0.01	0.34±0.01	0.78±0.01
C18:3n6	0.14±0.01c	0.06±0.01e	0.11±0.01c	0.09±0.01d	0.15±0.01c	0.12±0.00c	0.72±0.01a	0.12±0.01c	0.33±0.0b
C18:3n3	1.66±0.01bc	1.32±0.01 c	1.46±0.01 c	1.19±0.01 d	1.77±0.01 b	1.42±0.01c	0.83±0.01 e	1.80±0.01b	4.86±0.0a
C18:4n3	1.06±0.01b	0.80±0.01c	0.87±0.01c	0.54±0.01e	0.69±0.01d	0.82±0.01c	0.43±0.01f	0.70±0.01d	1.45±0.0a

Table 3.6, continued

Fatty acids	Control	SP12.5%	SP25%	SP50%	SP75%	CL12.5%	CL25%	CL50%	CL75%
C20:0	0.45±0.01	0.32±0.01	0.37±0.01	0.35±0.01	0.44±0.01	0.46±0.01	0.25±0.01	0.37±0.01	0.91±0.01
C20:1n9	2.96±0.01	2.94±0.01	2.84±0.01	2.23±0.01	2.00±0.01	2.69±0.01	1.32±0.01	1.94±0.01	4.40±0.01
C20:2	0.20±0.01	0.17±0.01	0.17±0.01	0.13±0.01	0.21±0.01	0.22±0.01	0.11±0.01	0.18±0.01	2.94±0.01
C20:3n6	0.10±0.01b	0.05±0.01bc	0.05±0.01bc	0.00±0.00	0.12±0.01b	0.07±0.01bc	0.00±0.00	0.05±0.01bc	0.23±0.01f
C20:3n3	0.75±0.01b	0.60±0.01bc	0.60±0.01bc	0.19±0.01de	0.66±0.01bc	0.64±0.01bc	0.25±0.01d	0.52±0.01c	1.10±0.01a
C20:5n3	5.93±0.01b	5.69±0.01bc	5.27±0.01c	3.60±0.01de	3.32±0.01e	5.24±0.01 c	3.40±0.01 e	4.35±0.01d	7.84±0.01a
C22:1n9	3.99±0.01	4.33±0.01	3.83±0.01	3.00±0.01	2.35±0.01	3.22±0.01	1.39±0.01	2.15±0.01	4.47±0.01
C24:0	0.37±0.01	0.37±0.01	0.39±0.01	0.26±0.01	0.26±0.01	0.35±0.01	0.06±0.01	0.19±0.01	0.62±0.01
C22:6n3	5.46±0.01b	5.21±0.01b	4.72±0.01c	2.74±0.01e	2.39±0.01ef	4.23±0.01e	27.72±0.01a	0.60±0.01f	3.75±0.01d
∑NESFAs	4.98±0.01	3.61±0.01	6.3±0.02	4.47±0.01	6.00±0.02	4.1±0.01	4.77±0.01	4.41±0.01	9.18±0.02
∑NEMUA	1.96±0.01	1.29±0.01	1.23±0.01	1.17±0.01	2.18±0.01	1.56±0.01	0.84±0.01	1.43±0.01	3.36±0.01
∑NEPUFs	0.68±0.01	0.59±0.01	0.57±0.01	0.32±0.01	0.39±0.01	0.51±0.01	2.5±0.01	0.14±0.01	0.61±0.01

Values are means of triplicates of nine different feed samples. Mean values on the same row (a - g) with different subscript are significantly different same ($P<0.05$). Saturated fatty acids consist of: 4:0, 6:0, 8:0, 10:0, 11:0, 12:0, 13:0, 14:0, 15:0, 16:0, 18:0, 20:0, 22:0, and 24:0. Monounsaturated fatty acids comprise: 14:1, 15:1, 16:1, 18:1n9c, 18:1n7, 20:1n9, C22:1n9 and 24:1n9. Polyunsaturated fatty acids consist of: 16:2n4, 16:3n4, 16:4n1, 18:2n6t, 18:2n6c 18:3n4, 18:3n6,18:3n3, 18:4n3, 20:2, 20:3n6, 20:3n3, 20:5n3, C22:2, and 22:6n3 n-3 fatty acids were: 18:3n3, 18:4n3, n-6 fatty acids: 18:2n6t, 18:2n6c, 18:3n6 n-3 Long chain polyunsaturated fatty acids found were: 20:3n3, 20:5n3 and 22:6n3 n-6 Long chain polyunsaturated fatty acids include: 20:3n6

∑ NESFAs: Summation of non-essential saturated fatty acids

∑ NEMUFAs: Summation of non-essential monounsaturated fatty acids

∑ NEPUFAs: Summation of non-essential polyunsaturated fatty acids.

However, for immune response and oxidative stress enzymes studies, the experimental diets (Tables 3.7 and 3.8) were chosen based on the results obtained from an earlier growth study (Figure 4.1) where the optimum growth was achieved at 68.5% and 69.4% for SP and CL respectively. Therefore, a total of five experimental diets were formulated, 35- 36% crude protein and 8% crude lipid. In each case based on the protein requirement of clariid fish juveniles, the diets were prepared in sets of either *Chlorella* (CL) or *Spirulina* (SP) powder to replace fishmeal at various inclusion levels of 0%, 50%, and 75% designated as control, SP50% / SP75% or CL50% / CL75% respectively. Amino and fatty acids composition of experimental diets are presented in Tables 3.9 and 3.10 respectively.

Table 3.7: Gross composition (g $100g^{-1}$ Dry Matter) of the Immunity experimental diets containing graded levels of *Spirulina* and *Chlorella*.

Proximate composition (%)					
Crude protein	36.11	36.41	36.06	36.07	35.56
Crude lipid	8.67	8.25	8.39	8.27	8.42
Ash	9.95	9.94	9.98	9.95	9.87
Moisture	9.32	8.99	8.95	8.91	8.47
Fibre	1.94	1.99	2.02	1.95	2.52
NFE	34.01	34.42	34.60	34.85	35.16
Gross energy Kcalg[1]	421.99	421.36	421.42	421.31	421.10
Dietary Treatments					
	CTR	**SP50**	**SP75**	**CL50**	**CL75**
Fishmeal	250	125	62.5	125	62.5
SBM	440	440	440	440	440
Corn Meal	100	100	100	100	100
Spirulina		125	187.5		
Chlorella				125	187.5
Vitamin Premix [a]	15	15	15	15	15
Mineral Premix [b]	15	15	15	15	15
Methionine	10	10	10	10	10
Lysine	10	10	10	10	10
Fish Oil	18	18	18	18	18
Binder	152	152	152	152	152
	1000	1000	1000	1000	1000

Each value is the mean of three replicates. Control (0% algae); SP50, SP75, CL50 and CL75 = 50% and 75% *Spirulina* and *Chlorella* meals respectively.

[a] Vitamin premix supplied: vitamins A,500IU; B1,1.0mg; B2,0.5mg; B3,0.3mg; B6, 0.2mg; B12,0.001mg; C,0.1mg D3 100IU; E,0.75mg, K,0.02mg; niacin,0.2mg, folic acid,0.1mg; biotin,0.24mg; pantothenic acid,1.0mg; inositol, 2.5mg

[b] Mineral premix provided the followings per kg diet: iron, 8.0mg; selenium, 0.2mg; magnesium oxide, 0.6 mg; manganese, 1.0 mg; zinc, 8.0 mg; copper, 0.15mg; potassium chloride, 0.4 mg; sodium bicarbonate, 1.5 mg; iodine,1.0 mg; cobalt, 0.25 mg)

Table 3.8: Gross composition (g/100g Dry Matter) of the Oxidative stress enzymes study diets containing graded levels of *Spirulina* and *Chlorella*.

	Dietary Treatments				
	Control	**SP50**	**SP75**	**CL50**	**CL75**
Fishmeal	25	12.5	6.25	12.5	6.25
SBM	43	43	43	43	43
Corn Meal	10	10	10	10	10
Spirulina		12.5	18.75		
Chlorella				12.5	18.75
Vitamin Premix [a]	1.5	1.5	1.5	1.5	1.5
Mineral Premix [b]	1.5	1.5	1.5	1.5	1.5
Methionine	1	1	1	1	1
Lysine	1	1	1	1	1
Fish Oil	1.8	1.8	1.8	1.8	1.8
Binder	15.2	15.2	15.2	15.2	15.2
Total	100	100	100	100	100
Nutrient level determined by as is basis (% dry matter basis)					
Crude protein	36.57	35.87	35.52	35.53	35.01
Crude lipid	8.21	7.71	7.85	7.73	7.87
Ash	9.55	9.94	9.98	9.95	9.87
Moisture	9.22	8.99	8.95	8.91	8.47
Fibre	1.94	1.99	2.02	1.95	2.52
NFE	34.51	35.5	35.68	35.93	36.26
Gross energy Kcal g^1	422.27	417.536	417.594	417.538	417.243

Each value is the mean of three replicates. Control (0% algae); SP50, SP75, CL50 and CL75 – 50% and 75% *Spirulina* and *Chlorella* meals respectively.

[a] Vitamin premix supplied: vitamins A,500IU; B1,1.0mg; B2,0.5mg; B3,0.3mg; B6, 0.2mg; B12,0.001mg; C,0.1mg D3 100IU; E,0.75mg, K,0.02mg; niacin,0.2mg, folic acid,0.1mg; biotin,0.24mg; pantothenic acid,1.0mg; inositol, 2.5mg

[b] Mineral premix provided the followings per kg diet: iron, 8.0mg; selenium, 0.2mg; magnesium oxide, 0.6 mg; manganese, 1.0 mg; zinc, 8.0 mg; copper, 0.15mg; potassium chloride, 0.4 mg; sodium bicarbonate, 1.5 mg; iodine,1.0 mg; cobalt, 0.25 mg).

Table 3.9: Amino acid profile of the experimental diets containing graded levels of *Spirulina* and *Chlorella*

	Dietary Treatments					**
	CTR	**SP50**	**SP75**	**CL50**	**CL75**	
Hydroproxiline	3.06 ± 0.01	3.54± 0.02	3.79± 0.02	3.29± 0.03	3.41± 0.01	
Aspartic acid	1.78± 0.01	1.89± 0.02	1.94± 0.04	1.76± 0.02	1.74± 0.01	
Serine	4.86± 0.01	5.15± 0.02	5.30± 0.17	4.88± 0.03	4.89± 0.03	
Glutamic acid	3.09± 0.02	3.40± 0.12	3.55± 0.02	3.39± 0.02	3.53± 0.01	
Glycine	0.95± 0.01	0.91± 0.02	0.90± 0.01	0.92± 0.01	0.91± 0.02	
Histidine*	2.47± 0.01 [c]	2.62± 0.01 [b]	2.69± 0.03 [a]	2.43± 0.02 [c]	2.41± 0.02 [c]	1.2[1]
Arginine*	1.54± 0.02 [b]	1.66± 0.03 [a]	1.72± 0.02 [a]	1.55± 0.02 [b]	1.55± 0.01 [b]	3.6 [1]
Threonine*	1.53± 0.01 [c]	1.79± 0.03 [b]	1.92± 0.01 [a]	1.80± 0.06 [b]	1.94± 0.02 [a]	2.80 [1]
Alanine	1.71± 0.03	1.74± 0.02	1.75± 0.02	1.80± 0.12	1.85± 0.02	
Proline	3.16± 0.02	3.65± 0.02	3.89± 0.02	3.40± 0.17	3.52± 0.01	
Cysteine	0.29± 0.02	0.27± 0.02	0.26± 0.02	0.22± 0.01	0.18± 0.01	
Tyrosine	1.11± 0.01	1.22± 0.01	1.28± 0.02	1.09± 0.02	1.08± 0.01	
Valine*	1.71± 0.02[d]	1.94± 0.02[b]	2.06± 0.02 [a]	1.85± 0.01[c]	1.92± 0.01 [b]	2.40 [1]
Methionine*	1.53± 0.02 [a]	1.50± 0.06 [a]	1.48± 0.02 [a]	1.48± 0.02 [a]	1.46± 0.02 [a]	2.3[*b]
Lysine*	3.32± 0.02 [b]	3.27± 0.02 [b]	3.24± 0.02[b]	3.41± 0.01 [a]	3.46± 0.02 [a]	4.80 [1]
Isoleucine*	1.51± 0.01	1.74± 0.02 [b]	1.86± 0.01 [a]	1.56± 0.02 [c]	1.59± 0.02 [c]	2.0 [1]
Leucine*	2.68± 0.02 [b]	2.90± 0.17 [ab]	3.01± 0.03 [a]	2.85± 0.02 [ab]	2.94± 0.02 [ab]	3.50 [1]
Phenylalanine*	1.63± 0.02 [c]	1.73± 0.02 [a]	1.79± 0.02 [a]	1.68± 0.02 [b]	1.70± 0.03 [b]	4.00 [*a]
Tryptophan*	0.35± 0.01 [a]	0.34± 0.02 [a]	0.34± 0.01 [a]	0.34± 0.01 [a]	0.33± 0.01 [a]	0.5[2]

Values are means of triplicates of five different feed samples. Mean values on the same row with unlike subscript are significantly different (P<0.05). Control (0% algae); SP50, SP75, CL50 and CL75 – 50% and 75% *Spirulina* and *Chlorella* supplemented meals respectively. *: Essential Amino Acids [1] Essential amino acid requirement (Jimoh *et al.*, 2014) [2] The essential amino acid requirement of *C. gariepinus*. Source: (Uys, 1989); (Unprasert, 1994) [*b] Methionine + Cysteine [a*] Phenylalanine/TAA (Total Amino Acid)

Table 3.10: Fatty acid profile of the experimental diets containing graded levels of *Spirulina* and *Chlorella*

	Dietary Treatments				
	CTR	**SP50**	**SP75**	**CL50**	**CL75**
SFA	42.55±0.06 ^{b}	40.73± 0.06 ^{e}	42.76± 0.05 ^{a}	41.56± 0.06 ^{c}	41.06± 0.02 ^{d}
MUFA	6.43± 0.12 ^{c}	6.35± 0.03 ^{bc}	6.79± 0.02 ^{a}	6.67± 0.12 ^{ab}	6.79± 0.02 ^{a}
PUFA	20.02± 0.06 ^{e}	21.92± 0.05 ^{b}	23.86± 0.02 ^{a}	20.78± 0.02 ^{d}	21.15± 0.02 ^{c}
∑n-6	9.99± 0.03 ^{e}	11.17± 0.02 ^{b}	12.07± 0.02 ^{a}	10.25± 0.02 ^{d}	10.38± 0.02 ^{c}
C18:3 n-4	0.58± 0.02^{c}	1.88± 0.03 ^{b}	2.58± 0.03 ^{a}	0.60± 0.12^{c}	0.62± 0.06 ^{c}
C20:5 n-3	1.04± 0.02^{c}	1.08± 0.02^{bc}	1.16± 0.02 ^{a}	1.11± 0.01 ^{ab}	1.15± 0.02 ^{a}
C22:6 n-3	0.88± 0.03 ^{d}	0.97± 0.02 ^{c}	1.05± 0.02 ^{c}	1.36± 0.02 ^{b}	1.61± 0.03 ^{a}
∑n-3	5.36± 0.02 ^{b}	5.00± 0.29 ^{b}	5.15± 0.02 ^{b}	5.87± 0.02 ^{a}	6.12± 0.01 ^{a}
∑n-9	3.99± 0.05 ^{a}	3.74± 0.02 ^{b}	3.90± 0.06 ^{a}	3.96± 0.02 ^{a}	3.94± 0.02 ^{a}
n-3/n-6 ratio	0.54± 0.02 ^{a}	0.45± 0.02 ^{b}	0.43± 0.01 ^{b}	0.57± 0.02 ^{a}	0.59± 0.03 ^{a}

Values are means of triplicates of five different feed samples. Mean values on the same row with unlike subscript are significantly different (P<0.05). Control (0% algae); SP50, SP75, CL50 and CL75 – 50% and 75% *Spirulina* and *Chlorella* meals respectively). PUFA: Polyunsaturated fatty acids; MUFA: Monounsaturated fatty acids; SFA: Saturated fatty acids, EPA: eicosapentaenoic acid and DPA: docosahexaenoic acid: ∑n-6 (comprising C18:2 n-6t, C18:2 n-6C, C18:3 n-6 and C20:3 n-6): ∑n-3 (C18:3 n-3, C18:4 n-3, C20:3 n-3, C20:5 n-3 and C22:6 n-3): ∑n-9 (C18:1n-9c, C20:1n-9, C22:1 n-9 and C24:1 n-9)

Diets were formulated using Pearson's square and WINFEED software version 2.8 Winfeed (UK) Limited Cambridge UK (Mirza, 2004). All dried feed materials were ground in a hammer grinder (Disk mill FFC 454, thereafter, vitamins, minerals, di-calcium phosphate (DCP and fish and palm oil (1:3) were gradually added and thoroughly mixed with water until a suitably textured dough was obtained. The dough was put in a fish extruder (16 mm die) and broken gently into 1 mm diameter pellets. Fish feed extrusion facility of BPTAP was used). The pellets so made were dried at 60^{o} C for 24 hours in an oven; they were later packed in polypropylene bags, which had been earlier labelled, after cooling to room temperature. The bags were sealed and stored at 4^{o}C in a cold room for feeding trial in the future.

3.4.2 Experimental setup and fish

The study was carried out inside the aquarium research laboratory of the Institute of Biological Sciences, Faculty of Sciences, the University of Malaya, Malaysia. The pre-experiment activities involved the procurement of fish from Salak-Tinggi research station in Malaysia. All experimental fish were familiarized to the condition of the experiments for 2 weeks before the commencement of the experiment. During this period, they were given commercial feed. For digestibility study, the pre-experiment activities involved the procurement of 120 fish with a mean weight of 58.05 g, out of which 90 fish were randomly divided into three different groups, replicated thrice with 10 fish per replicate (tank). The three experimental diets (reference and the two test diets) were randomly distributed to the nine experimental tanks (three replicate tanks/diet). For a week, they were Fed with their three allotted diets ad-libitum to familiarise them to the feeds before the faecal collection. Thereafter, fish were Fed two times daily at the rate of 4% of their body weight between 0800 and 1700hr daily for 42 days. The uneaten feed was removed within an hour interval to avoid contact with faecal samples. For growth

performance study, 270 fingerlings mean weight (7.85 g) were randomly stocked into 27 different tanks, with 10 fish per tank and three replicates per treatment. Experimental fish were Fed twice daily between 900 hours and 1600 hours at 4% body weight for 56 days. On the commencement of the feeding trials, 10 fish samples were homogenized then frozen while three per replicate were sacrificed by putting them in water containing clove oil (40mg l^{-1}), for subsequent carcass examination at the completion of the experiment. For immunity and oxidative stress studies, 275 fingerlings mean weights 41.86 ± 0.02 and 42.07± 0.02 g respectively were randomly stocked into 15 different tanks, fifteen fish per replication with 3 replicates per treatment. Experimental fish were Fed twice daily at both 3% (for former) and 2% (for later) body weight for 16 weeks to study the effect of long-term feeding of both algae on growth immune parameters and oxidative stress enzymes.

3.4.3 Faeces collection

Faeces were collected through syphoning from the bottom of the tanks (Ogino *et al.*, 1973) twice daily before feeding in the morning and before feeding in the afternoon, for 42 days and stored at -20 ºC. Tanks pooled faecal samples for the period of the study were finally dried in an oven and finely ground. They were then stored at -20 ºC for proximate fatty and amino acid analysis (FA and AA).

3.4.4 Water quality measurements

The experimental tanks used for all the studies were of 150 L capacity with a close re-circulation system. They were equipped with top filter pumps (H6350, Shanda, Shanda Aquarium, China) and connected to filter boxes (for continuous syphoning of uneaten feed and faeces) with a flow rate of 20 L $^{-1}$min and aeration diffuser (Sonic air pump P85, China) for the flow of dissolved oxygen. The tanks were washed, disinfected

dried and filled with de-chlorinated water to two-thirds of the experimental tank volume, then covered by a net of mesh size 2.0 - 3.0 mm to protect the fish from jumping out. To maintain water quality excellent condition for catfish, 30, 50 and 100% of water volume was replaced at daily-, two-day and weekly intervals. Three tanks were used for each treatment. Water quality was monitored regularly according to APHA (1992). With the aid of Extech DO700 meter (Extech FLIR Systems, USA), dissolved oxygen was measured and maintained above 4.0 mg/L at temperatures 26 - 27 °C and pH at 6.0 - 6.8. Nitrate and ammonia were determined weekly according to Marion (1998) and maintained at < 1.9 mg^{-1} and < 0.80 mgL^{-1} respectively.

3.4.5 Proximate and chemical examination

The diets, faucal and fish samples used for the experiment were sent to the University Kebangsaan Malaysia (UKM UNIPEQ) feed laboratory, Malaysia for proximate and amino acid composition, while fatty acid was analysed at the University of Malaya algae research laboratory by the method of Association of Analytical Chemists (AOAC, 2000). The AOAC (2005) official analysis methods were followed in the analysis of the experimental feed and faeces to analyse their proximate compositions.

3.4.6 Crude protein analysis

Samples crude protein was obtained after determining the nitrogen by acid digestion using Kjeldahl apparatus (Vapodest 50 Gerhardt Germany), based on N × 6.25 (method 981.10). Briefly, 150 mg of feed/ faecal samples were weighed into Kjeldahl digestion flask and a tablet (100 mg) Selenium Kjeldahl tabs (catalyst) plus 6ml sulphuric acid were added. These were digested at 420 ^{o}C for an hour. The digested samples were left to cool for 15 minutes before distillation. The titration indicator was prepared by dissolving 100 mg of bromo cresol green in 100 ml methanol, also, 70 ml methyl red

solution in 100ml methanol. Both solutions were then mixed together. Thereafter, 80 ml of de-ionized water and 50 ml of sodium hydroxide (NaOH) were added to each digestion flask, mixed thoroughly, distilled with 25 ml of 4% boric acid as well as titration indicator. 0.01 M Merck hydrochloric acid was used to titrate the product of distillation in the flasks and titer values recorded automatically. All these were done in the programmable unit of the Vapodest50 equipped with a titrator with automatic addition of water, NaOH and boric acid. All blanks and samples were analyzed in triplicates. Data are processed with Vapodest Manager Software and the crude protein was calculated automatically as:

$$\% \text{ Nitrogen} = (S - B)(N)(14.007) * 100 \div (B) * 1000 \quad 3.1$$

$$\text{Protein} = (\%Nitrogen) * (6.25) \quad 3.2$$

Where S = Titrate of HCl (ml), B = sample /blank (g), N = HCl molar (0.0994)

3.4.7 Crude lipid analysis

The Bligh and Dyer (1959) method, with slight modifications, was used to quantify the lipid contents of *Spirulina* and *Chlorella* while the petroleum ether extraction method was used for other samples. This involved either dissolving the samples (*Chlorella* and *Spirulina*) in a mixture of 2:1 chloroform and methanol in Soxhlet apparatus (Gerhardt Soxtherm) for 8 hours or in petroleum ether for1.5 hr (other samples). Briefly, extraction cups were dried in an oven and weighed. Thereafter, 2g of samples were weighed and added into cellulose thimbles and extraction cups were then filled with 80 ml of petroleum ether for other samples (method 985.01) or a mixture of chloroform and ethanol at the ratio of 2:1 (for *Spirulina* and *Chlorella*). Both thimbles and extraction cups were placed in the extraction unit of Gerhardt Soxtherm, and the extraction process was done for 1.5hr (other samples) or eight hours for *Spirulina* and *Chlorella*. Afterwards,

the extracted oil and cups were dried in an oven at 120 ºC for 2 hours, removed and cooled off in a desiccator before weighing. Samples residue in the thimbles were later stored in the desiccator and used for crude fibre analysis. Samples were all analyzed in triplicates. Chloroform and methanol mixture was used for both algae as the researcher found that petroleum ether did not extract all the oils in both algae.

The lipid contents of the sample were calculated per the equation below as:

$$\%\ Lipid\ = \frac{(W3-W2)}{(W1)} X100 \qquad 3.3$$

Where W1 = Sample weight (g), W2 = Initial weight of extraction cup (g), W3 = Final weight of extraction cup (g)

3.4.8 Dry matter measurement

Dry matter and moisture were determined gravimetrically through oven drying in (Memmert 500 Germany) at 105ºC to a constant weight (method 934.01). This involved drying and weighing of empty crucibles to obtain their initial weight. Approximately 4 g of samples were placed in the crucibles, weighed and recorded prior drying in the oven for 24 hours to a constant weight. The residues were later cooled in the desiccator and re-weighed to obtain final weight. The sample dry matter content was obtained by the equation below:

$$\%\ Dry\ matter\ = \frac{(W3-W1)}{(W2-W1)} X100 \qquad 3.4$$

Where W1 = Empty crucible weight, W2 = Crucible + Sample weight, W3 = Crucible + sample weight after drying at 105 ºC

3.4.9 Ash determination

To determine the ash contents both diets and flesh composition, the dry matter residue was ignited (combustion) in a muffle furnace (Memmert UFB500 and Carbolite Furnace Memmert CWF 11/13 Germany) at 600 ºC overnight (method 942.05). The sample residues were then cooled in a desiccator and reweighed to determine the ash content. The ash content was calculated from the equation below:

$$\% Ash = \frac{(W4 - W1)}{(W3 - W1)} X100 \qquad 3.5$$

Where W1 = Weight of empty crucible, W3 = Weight of crucible + sample after drying at 105 ºC, W4 = Weight of crucible + sample after drying at 600 ºC

3.4.9.1 Determination of samples crude fibre

Samples crude fibre contents were determined by alkali and acid digestion of defatted lipid residue (method 962.09). Fibre capsules together with their lids were weighed individually. Approximately 50-100 mg were then weighed in a filter paper and put in the capsules and covered. Then 350 ml of 1.25% (v/v) sulphuric acid was placed in a hot pot and heated to boiling point. Thereafter, the capsules + samples were placed in the carousel with a stopper to lock the capsules in place. These were then moderately lowered into the boiling reagent adequate to immerse the samples. The samples were gently boiled for 30 minutes. After 5 minutes of boiling, the carousel was removed from the extraction vessel and washed in boiling water 3 times with fresh hot water each time. Thereafter, the vessels were filled with 350 ml 1.25% (w/v) NaOH and boiled in a hot plate and followed by washing as previously done for sulphuric acid. Samples were later washed in 1% (v/v) HCl followed by in clean plain water and dried in an oven at 130 ºC for 2 hours, cooled in a desiccator and weighed. The weighed samples were then placed in a pre-dried and pre-weighed crucible for ashing at 600 °C for 4 hours and cooled in a

desiccator prior to re-weighing to determine the crude fibre content. The crude fibre content was calculated using the equation below:

$$Crude\ fiber\ = \frac{W3 - (W1XC) - (W5 - W4 - D)}{W2} X100 \qquad 3.6$$

Where W1 = Initial capsule weight (mg), W2 = Sample weight (mg), W3 = Capsule + residue weight, W4 = Initial weight of crucible (mg), W5 = Total ash (g), C = Blank correction for capsule solubility, D = capsule ash (mg).

3.4.9.2 Determination of sample gross energy contents

Energy is required for catfish to grow and achieve some higher levels of outputs. As such appropriate measurement of gross energy required in catfish diet is calculated with the combination of other nutrient components. Jobling (1983) presented the combination as:

$$Energy\ (Kcal\ /kg\ = \text{CP x } 5.65 + \text{lipid x } 9.45 + \text{carbohydrates x } 4 \qquad 3.7$$

3.4.9.3 Nitrogen free extract (NFE)

NFE was calculated as =100- (% crude lipid+ % crude fibre + % crude protein + % crude ash).

3.4.9.4 Chromic oxide determination

After nitric acid digestion of feeds and faecal samples in a laboratory microwave digester (Milestone, MLS1200 Mega, Italy), with the use of inductively-coupled-plasma mass spectrophotometry (ICP-MS) (Perkin Elmer ELAN 9000ICP/M System USA), the concentrations of chromium in the faecal materials and diets were analysed. This was in accordance with the specifications of (AOAC, 1992) (Methods 968.08 and 965.09) as described by (Li *et al.*, 2013) and ICP-MS 9000 Operator manual. Briefly, 0.5g each of

dried faecal and feed samples was weighed, and place in a Tetrafluor Methoxyl (TFM) vessel, 9 ml of 65% ultra-pure nitric acid ($HN0_3$) (Merck) and 2 ml of 30% hydrogen peroxide were added. The vessel was left to react for a minute before sealing and placed in the polypropylene rotor body and later placed in pairs opposite each other. The screws of the polypropylene rotor body are then tightened with a wrench to make sure the vessels are locked and placed in the microwave digester. The following irradiation programme was selected: 250 W for 1min, 0W for 2 min, 250 W for 5 min, 400W for 6 min and 650W for 6 min. Rotor and vessels were cooled for 10 minutes, then dried and uncapped slowly in the fume hood until the pressure is completely released. The digested samples were then transferred into polypropylene disposable tubes and made up to 50 ml with ultrapure water. Each sample was digested in triplicates, and two water blanks were run with the samples. Chromium oxide was analysed with ICP-MS at 205.563 nm (Canbay & Doğantürk, 2017). The concentration of Cr_2O_3 was calculated by the equation below:

$$Concentration\ in\ ppm = \frac{\mathrm{Re}ading\ x\ volume\ diluted}{Sample\ weight} \qquad 3.7$$

3.4.10 Quantification of feed and faecal amino acids

The essential amino acid composition of freeze-dried feed and faeces was measured using high-performance liquid chromatography (HPLC), fortified with a fluorescence detector (Taufek *et al.*, 2016) and the contents were quantified using the Pico-Tag technique (Heinrikson & Meredith, 1984). The alkaline hydrolysis method was used to determine tryptophan (Nielsen & Hurrell, 1985).

3.5 Preparation of samples

Samples were ground, dried and analysed for crude protein prior to being used for amino acid analysis. This is to determine the amount of the samples to be used, as the crude protein of each sample determines its amount to be used for amino acid analysis. The weight of each sample to be measured was gotten by dividing 4 with the crude protein content of the sample. For example, the crude protein of *Spirulina* used for this study at 66.44%, thus 4/66.44 = 0.06 g that was used for the analysis. The weighed samples were hydrolyzed with 6 N HCl and vortexed. Then flushed with N2 gas for a minute and oven dried at 140 ^{o}C for 24 hours, cooled off at room temperature. A 10-m internal standard, α-amino-N butyric acid (AABA) was added to the samples and made up to up with 60 ml of de-ionized water. Samples were later filtered and kept in -20 ^{o}C for further analysis. The internal standard was then prepared by dissolving 0.2578 g AABA and made up to 1 L of 0.1 N HCl.

3.5.1 Drying and derivation procedure

The re-drying agent was prepared from methanol-water and triethylamine at the ratio of 2:2:1 v/v while derivation agent composed of methanol, triethylamine, water phenylisothiocyanate (PITC) at (7:1:1:1 v/v). Hydrolyzed samples were then filtered with 0.20 µm cellulose nitrate membrane filter and 10µl of the samples were placed in a vial.

HCl from the samples was removed by vacuum and dried at room temperature for 30 minutes, before re-vacuuming with 20µl re-drying agent. This is followed by the addition of derivatization reagent and vortexing. Samples were left to stand at room temperature for 20 minutes before re-vacuuming again for 30 minutes. This was done to ensure that the reagents are completely dried.

3.5.2 Chromatographic procedure

The high-performance liquid chromatography (HPLC) was used to titrate PITC derivatives. Agilent Technologies columns were used for this process. The mobile phase consisted of two eluants labelled A and B. Solvent A consisted of 0.1 M ammonium acetate while solvent B composed of 440 ml solvent A mixed with 460 ml acetonitrile and 100 ml methanol. Both eluants were filtered and degassed and kept at room temperature. Thereafter, samples and standard were mixed with 100µl of solvent A and vortexed for 15 minutes before injecting into HPLC machine. The amino acids of the samples were quantified by comparison of peak retention times to the known standard.

3.5.3 Determination of tryptophan

The tryptophan contents of the samples were hydrolyzed by 4.3 N Lithium hydroxide (LiOH) (Nielsen & Hurrell, 1985) using a fluorescence detector using the excitation and emission wavelength given. Briefly, tryptophan standard was prepared by weighing 0.05 g of tryptophan into 50 ml volumetric flask. HCl (0.1N) was added to the flask and placed into ultrasonicator to dissolve the solute. A total of 50 ml of distilled water was added to the solution to make a 1000 µg/ml tryptophan concentration. Subsequently, 50 µl of the solution was added to 10 ml mobile phase to make 50 µg/ml while 10 µl (tryptophan standard) was injected into the HPLC system.

On the other hand, Lithium hydroxide was prepared by dissolving 36.09 g $LiOH.H_2O$ in 200 ml dH_2O to measure up to 10 samples. Samples were prepared by adding 0.2 g of samples and 15 ml of $LiOH.H_2O$ together in a screw-capped tube. The solution was flushed with liquid N_2 and heated at 120 ºC for 16 hours. Then, the hydrolysate was transferred into a beaker, and 9 ml of 6 N HCl with dH_2O was added to make a total volume of fewer than 100 ml. The pH was then adjusted to 4.5 by using HCl and diluted to 100 ml with water into a volumetric flask. The reagent was later filtered

through filter paper while a small aliquot was filtered through a syringe filter (0.2 µm cellulose acetate membrane) out of which, 10 µl of these (sample aliquots) were injected into the HPLC system.

3.6 Feeds and faecal fatty acid (FA) determination

3.6.1 Determination of samples lipid composition

Lipid contents of dried feed samples and *Spirulina* and *Chlorella* powder was determined gravimetrically (Bligh & Dyer, 1959). Briefly, samples were weighed and placed in test tubes, centrifuged at 1,200×g for 10 minutes and dried at 51 ºC, 10×10^4 atm. Afterwards, freeze-dried samples were kept in a desiccator overnight to a constant weight. Subsequently, freeze-dried samples were ground with the aid of laboratory mortar and pestle to extract the lipids. Lipids were extracted in MeOH-$CHCl_3$ -H_2O at (2:1:0.8 v/v)

3.6.2 Transesterification of lipid and FAME analysis

The resultant lipids were transesterified in 1.2 % HCl in MeOH, toluene, and water (100 °C, 1 h) (Ichihara & Fukubayashi, 2010)Then, the extracted fatty acid methyl esters (FAME) were stored in an inert atmosphere (N_2) in a freezer at −20°C.The FAME composition of the individual samples were evaluated by using Agilent 7820 A gas chromatograph fitted with a capillary column (SLB-IL 100, 30 m × 0.25 × 0.20 µm, Supelco, USA) in addition to a flame ionization detector (FID) using the temperatures of injector and detector at 250 and 260 ℃ correspondingly.

The ensuing thermal program as reported by Vello *et al.* (2014) was used: 140 °C for 5 min, then increasing 8°Cmin $^{-1}$ up to 180 °C, following an increase of 5 °C min^{-1} up to 260 °C. Helium was used as carrier gas at 30.97 mL min^{-1}. Hydrogen gas and purified air flow were provided at 40 and 450 mL min^{-1}, respectively. The injections

were done two times for each extraction in a volume of 1µl. Different FAMEs were recognized by equating their retaining times with those of authenticated standards from (Sigma-Aldrich®, USA), Fatty acids were quantified in milligram per gram of lipids, by means of the addition of an internal standard C7:0 Sigma®, USA as reported by (Vello *et al*., 2014).

3.7 Data analysis

This section deals methods used to analyse the data obtained during experimental procedures as highlighted above.

3.7.1 Growth performance analysis

Feed intake was recorded, and uneaten feed removed one-hour afterwards and weighed to compute the amount of feed consumed. Fish sampling and weighing were done fortnightly. The quantities of feed administered to each tank were adjusted per the new weight. The tanks were monitored closely for mortality, and dead fishes were removed and recorded for determination of survival rate. The growth performance indices were computed following the method by Badwy *et al.* (2008) and Promya and Chitmanat (2011).

Mean weight gains (MWG) = $Wf - \frac{Wi}{n}$

Where: Wf: final weight; Wi: initial weight; and n: number of fish.

Relative growth rate = $\frac{Wg}{Wi} \times 100$

Where: Wg = weight gain and Wi = initial weight.

Specific growth rate

$$(\log Wf - \log Wi) \times \frac{100}{t}$$

Where: logWf = log of final, logWi= log of initial weight, and t = time.

Feed conversion ratio (FCR) = $\frac{Fi}{FWg}$

Where: Fi = dry feed Fed and FWg = fish wet weight gain.

Protein efficiency ratio = $\frac{MWG}{MPI}$

Where: MWG = mean weight gain and MPI = mean protein Fed.

Hepatosomatic index = $\frac{\text{Liver weight (g)}}{\text{body weight (g)}} \times 100\%$ (Nunes *et al.*, 2011)

Survival rate = $\frac{Fn}{In}$ x 100

Where the Fn = final quantity of fish at the end of the experiment and In = initial quantity of fish at the beginning of the experiment.

3.7.2 Calculation of apparent digestibility coefficient (ADC)

The ADC of nutrients, dry matter (DM), fatty and amino acids availability coefficient (AAAC & FAC) were estimated using the equations earlier described by (NRC, 2011)

ADC dry matter: $ADC_{DM} = 100 - \left(100 \times \left(\frac{Crdiet}{Crfeaces}\right)\right)$ 3.9

ADC for protein, carbohydrate, lipid, ash, fibre, amino and fatty acid digestibility coefficient as:

ADC for reference and test diets:

$$= 100 - \left(\frac{Crdiet}{Crfeaces}\right) \times \left(\frac{nutrient\ in\ feace}{nutrient\ in\ diet} \times 100\right) \quad \text{.....3.10}$$

Where ADC = Apparent digestibility coefficients, Cr = % Chromium (Cr_2O_3), DM = Dry matter and Nutrients = protein, lipid, ash, carbohydrate, fibre and amino and fatty acid.

3.7.3 Blood and serum collection. haematological and biochemical analysis

Fish were starved of feed 24h before sampling. To avoid the probable effect of stress on the analysed parameters, fish were anaesthetised with clove oil (40 mg L^{-1}) and were bled immediately after capture (Pickering *et al.*, 1982). Blood samples were collected from the caudal vein of three randomly selected fish from each tank with 1-ml syringes and 25-gauge needles into three different tubes (EDTA, heparinized and serum). Parts of the samples (the first two) were immediately used for determination of haematological parameters (complete blood counts) and respiratory burst activity. Serum was obtained from the blood sample (third tube) after centrifuging at 5000 rpm for ten minutes and used for determination of biochemical parameters).

Complete blood count which consisted red blood cells (RBC), white blood cells (WBC), haemoglobin concentration (HGB), platelets, haematocrit (HCT) and red blood cell distribution width (RDW) were measured with an automatic haematology analyzer (Sysmex XN, Germany). Similarly, RBC indices namely: mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentrations (MCHC) were also calculated automatically with the same analyzer. Biochemical parameters namely glucose, total serum protein, serum albumin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT),

creatinine and lipid profiles (serum triglycerides, total cholesterol, HDL and LDL cholesterol) were measured with Advia 2400 Chemistry System (Siemens Healthineers, Germany). Haematological and biochemical parameters were performed prior to and after *A. hydrophila* challenge whereas respiratory burst activity was done post challenge. Haematological parameters (RBC indices) were determined based on Seiverd (1983).

$$\text{MCV (fl)} = \frac{\text{Hct}}{\text{Hgb}}$$

$$\text{MCH (pg.)} = \frac{\text{Hgb x 100}}{\text{RBC}}$$

$$\text{MCHC (gdl}^{-1}\text{)} = \left(\frac{\text{Hgb}}{\text{Hct}}\right) \text{x } 100$$

Where MCV = Mean corpuscular volume, MCH = Mean corpuscular haemoglobin and, MCHC = Mean corpuscular haemoglobin concentration, Serum globulin g dl^{-1} = Total protein – albumin

3.7.4 *Aeromonas hydrophila* challenge test

At the end of the feeding trial, 30 fish from each treatment (10 fish per replicate) were challenged with a virulent strain of *A. hydrophila* which was obtained from the Universiti Putra Malaysia (UPM) microbial repository. A preliminary challenge using a similar group of catfish Fed commercial feed and kept under same environmental conditions was performed to establish the LD_{50} dose of the bacterium. Original suspension of *A. hydrophila* was prepared by centrifuging an overnight culture (18h) of the bacterium. Briefly, a loopful of the bacterium was inoculated into tryptic soy broth and incubated for 18 h at 30 °C. The culture was centrifuged at 8000 rpm for 10 min at 4 °C and the supernatant was discarded. The cell pellet was washed twice in sterile phosphate buffer saline (PBS) solution (pH 7.4) and finally re-suspended in 25 ml fresh buffer.

Varying concentrations (2.5 x 10^0 – 2.5 x 10^6 cfu ml^{-1}) of this suspension were then prepared using 10-fold serial dilutions. Seventy fish were divided into seven tanks comprising 10 fish each and were challenged with the various dilutions. They were monitored for mortalities within 96 hours. The LD_{50} was found to be 2.5 x 10^6 cfu ml^{-1} from this experiment and was subsequently used in the actual challenge test. The fish were injected intra-peritoneally with 100 µL of the bacterial suspension 24 h after feed restriction. Feeding was resumed following the challenge. Mortalities were monitored for 18 days.

3.7.5 Confirmation of bacterial pathogenicity

To confirm that fish death was due to *A. hydrophila,* dead fish were immediately removed and dissected aseptically to remove the intestine and liver. These were then weighed and homogenised in sterile physiological saline (1:9). The bacterial load from each organ was then determined using the drop-plate technique (Herigstad *et al.*, 2001), following a series of serial dilutions. 100 µl from each of the serially diluted solution was spread onto selective media containing Mueller-Hinton agar supplemented with 5% defibrinated sheep blood and 30 µg/ml ampicillin, which was used as the growth medium (Misra *et al.*, 1989). Inoculated plates were incubated at 30 °C for 12 h.

3.7.6 Respirator burst activity assay

Blood samples were collected from surviving fish as described in section 3.7,3, ten and eighteen days post challenge. Leukocytes respiratory burst activity was determined following the method described by Anderson and Siwicki (1995) as modified by Kumari and Sahoo (2005). The method involved the colourimetric determination of oxidative radical produced by the leukocytes respiratory burst which promotes the reduction of nitro blue tetrazolium (NBT) (Sigma, St. Louis, MO, USA) into dark

precipitate inside the phagocytes called formazan granules. Briefly, 100 µl each of blood samples and 0.2% NBT were mixed together in equal proportion (1:1) homogenized and incubated at 25 °C for 30 min. The homogenate (50 µl) was dispensed into glass tubes and mixed with 1 ml of N, N-dimethylformamide (DMF) (Sigma Germany). This was centrifuged at 3000 g for 5 min. The optical density of the supernatant was measured at 540 nm with the aid of a microplate reader (Tecan Switzerland). The blank consisted of the same constituents except for blood which was replaced with distilled water.

3.7.7 Lysozyme activity assay

Serum lysozyme activity was determined (10^{th} and 18^{th}-day post challenge) turbidimetrically according to the method described by Zhou *et al.* (2006) with slight modifications. Lyophilised *Micrococcus lysodekticus* (0.75 mg/ml) (Sigma Aldrich Germany) was suspended in 100 mills sodium phosphate buffer (0.1 mM, pH 6.4). Exactly 20 µl of serum sample was added to 200 µl of the bacterial suspension in a 96-well plate which was incubated at room temperature for one and 30 min. The reduction in absorbance was measured at 570 nm in a plate reader (TECAN, Switzerland). One unit of lysozyme activity (U/ml) was expressed as the amount of enzyme producing a reduction in absorbance of 0.001/min during the 30-min incubation.

Lysozyme activity was calculated according to Shugar (1952) with slight modification as: Units/ml enzyme = (Δ_{570}//min Sample- Δ_{570}//min)/ (0.001) (0.02)

Where: df = Dilution factor, 0.00 1= $\Delta_{570\ as}$ per the Unit Definition, 0.1 = Volume in (milliliters) of enzyme solution.

3.7.8 Liver samples preparation

After 12 weeks of the experiment, five fish samples were randomly collected from each tank for liver analysis. Each of the fish samples was marked, recording their

body weights and lengths, before dissecting them to their anatomical parts, weighed and recorded appropriately. The livers and gonads were removed and weighed to determine the hepatic and gonad somatic index (HSI % and GSI %). Homogenization of liver was performed in 6 ml buffer using 0.6 g sample each. The buffer solution consists of 0.1 mM phenylthiourea (PTU), 0.1 mM dithiothreitol (DTT), 1.0 mM EDTA, 0.1 mM protease inhibitor, and 25 mM phosphate buffer (pH 7.4). The homogenates were centrifuged using Beckman 80Ti ultra-centrifuge at 100,000 × g for 30 minutes at 4°C and resultant supernatants were stored in -80 °C fridge for enzyme analysis as described by Taufek *et al.* (2016).

3.7.9 Liver protein concentration

The protein concentration of liver was obtained using the Bio-Rad DC colourimetric protein assay (Bio-Rad 500-01 16) on the basic reaction of protein with an alkaline copper tartrate in a two steps process leading to colour development.

(1) The interaction of the protein with copper in an alkaline solution and

(2) The successive reduction of Folin reagent using copper-treated protein (Lowry *et al.*, 1951; Peterson, 1977; Peterson, 1979).

The reagents package for this assay contains Reagent A (alkaline copper tartrate solution), REAGENT B (dilute Folin Reagent), and reagent Bovine serum albumin (Bio-Rad 500-0007 medium) were used as the standard. A 200 µl volume of reagent S was added to 10 ml of reagent A (working reagent A). A 10-mg ml^{-1} stock solution of Bovine serum albumin (BSA) was prepared, out of which eight standard solutions were formulated ranging from 0.2-1.6 mg ml^{-1} as shown in Appendix A. 5 µl each of standards and samples was pipetted into each well of a clean, dry 96 well plate. This was followed by adding 25 µl of reagent A and finally 200 µl reagent B. The plate was then placed in a

micro-plate reader (TECAN Switzerland). The mixture was left to stand for 15 minutes before absorbance was observed at 650 nm. The quantity of BSA in the samples was plotted against their corresponding mean absorbance (appendix B). The amount of protein in the samples was determined from the curve.

3.7.10 Oxidative stress assay

The CAT activity was assayed as described by Claiborne (1985). The reaction mixture comprises 50 mM Na_3PO_4 buffer at neutral pH and 19 mM H_2O_2 prepared by using a Na_3PO_4 buffer. 300 µl of H_2O_2, 50 µl of samples and 2.65 ml of Na_3PO_4 buffer were mixed in a cuvette in a 3-ml reaction mixture. The reaction was measured at 25°C by recording the consumption of H_2O_2 at 240 nm. The CAT activity was obtained as nmol H_2O_2 disappeared/min/mg protein (ε_{240nm} = 0.0436 m/M/cm) using equation below

$$\text{Enzyme activity: } \frac{(\frac{\triangle A240nm}{min} \text{x (sample} - \text{blank) x 3 x df)}}{0.0436 \text{ x sample (ml)}} \qquad 3.11$$

The SOD activity was determined as that adopted by Taufek *et al.* (2016). In the reaction mixture, the final concentration comprises 0.005 mM xanthine oxidase, 0.05 mM xanthine, 0.01 mM cytochrome c, 0.1 mM EDTA and 50 mM sodium phosphate buffer. The reaction commenced upon addition of xanthine oxidase to the enzyme extract at 25 °C and 550 nm absorption. The SOD activity is a measure of its ability to prevent 50% cytochrome c reduction, and the results were given as nmol/min/mg/protein as shown in the equation below:

$$\text{Percent inhibition: } \frac{(\triangle \frac{A550nm}{min} \text{x (XOD} - \text{sample) x 100)}}{\triangle \frac{A550nm}{min} \text{x (XOD} - \text{blank)}} \qquad 3.12$$

$$\text{Specific activity } \frac{(\%InhibitionXdf)}{50\% \, Xsample(ml)Xsample\ (protein)} \qquad 3.13$$

The GST activity was determined by obtaining its response to 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm (Taufek *et al.*, 2016). The assay comprises 60 mM CDNB (dissolved in ethanol), 60 mM glutathione (GSH), and 100 mM sodium phosphate buffer (pH 6.5). The activity of the GST was obtained as the quantity of enzyme that catalysed the conjugate of GSH per min and 1 µmol of CDNB at 25 °C (ε_{340nm} = 9.6 mM^{-1} cm^{-1}), which was obtained as nmol/min/mg protein as shown in the equation below.

$$\text{Enzyme activity: } \frac{\left(\Delta\frac{A340nm}{min}\text{x (sample} - \text{blank)x 3 x df}\right)}{9.6 \text{ x sample (ml)}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 3.14$$

$$\text{Specific activity (nmol): } \frac{Enzyme\ activity}{protein\ concentration\ of\ the\ sample} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 3.15$$

3.7.11 Statistical analysis

With the use IBM SPSS Statistics for Windows, Version 21.0 (IBM Corp, Armonk, New York) the data obtained from ADC and growth values were analysed using one–way analysis of variance (ANOVA). Two-way ANOVA (2 × 5 and 2 × 3) models were used to determine the effect of algae on a dietary level based on the interaction between algae and dietary level on growth, haematological and biochemical parameters as well immunological responses of *C. gariepinus* in the study respectively. Significant parameters for dietary level were further analysed using Duncan multiple pairwise comparisons to determine the significant pairs. Paired t-test was used to compare the means of pre and post-challenge haemato-biochemical parameters as well as days 10^{th} and 18^{th} lysozyme and RBA activities. Curve estimation (polynomial) regression analysis was done with the aid of Wolfram Mathematical 11 version 11:0.1.0 student edition (Wolfram Research Inc, Champaign IL, USA) using equations $Y = -0.001X^2 + 0.137X + 11.191$ and $Y = -0.0011X^2 + 0.1527X + 11.298$ to estimate the optimum level of dietary replacement with *Spirulina* and *Chlorella* at different percentage compositions based on

weight gain of *C. gariepinus* fingerlings respectively. The probability level of the differences between means was calculated at 5% ($P < 0.05$). Data were presented as means ± SE (standard error).

CHAPTER 4: RESULTS

4.1 Nutrients, amino and fatty acids digestibility of dietary *Spirulina*, *Chlorella* and Fishmeal in African catfish (*C. gariepinus*)

4.1.1 Proximate compositions of feed ingredients used for experimental diets

Proximate amino and fatty acids compositions feed materials used for the experimental diets are presented in Appendices A, B and C. *Spirulina* and *Chlorella* powder contained 66.44 ± 0.37 and 14.68 ± 0.37, 58.84±2.64 and 18.29 ± 0.93 of protein and lipid respectively. Both algae were rich sources of amino acids (Appendix B). Alanine, leucine proline, methionine, lysine, and phenylalanine were significantly higher ($P < 0.05$) in *Chlorella* while *Spirulina* was significantly better in arginine, threonine, tyrosine valine aspartic acid, serine, glutamic acid, isoleucine, and glycine. Histidine plus hydroxyproline was absent in both algae while cysteine was lacking in *Chlorella* in this study. Similarly, the fatty (FA) acid profile as shown in Appendix C indicated that *Chlorella* had significant parts of its total FA characterised by Mono Unsaturated FAs Fatty Acids (MUFA), palmitoleic acid (C16:1), oleic acid (C18:1n9), butyric acid (C4:0 stearic (C18:0 linoleic (C18:2n6) alpha-linolenic (C18:3n3) and lignoceric (C24:0) acids, while *Spirulina* shows significantly higher contents of Saturated Fatty Acids (SFA), Palmitic (C16:1) gamma-linolenic (C18:3n6) and homo-y-linolenic (C20:3n6) acids.

4.1.2 Proximate amino and fatty acids composition of reference and test diets

Table 3.1 shows that all the protein contents of the diets were 39.38 - 41%, iso-energetic at 442 Kcal 100 g^{-1} and with fibre content range of 0.8- 1.21 %. The reference diet had a higher crude lipid (8.81%) and ash (8.69%) while *Chlorella* diet had the highest carbohydrate (34.79%).

All the EAAs were present in all the tree diets as shown in Table 3.2. Lysine was the most abundant while tryptophan was the least EAAs in both test diets. Except for the values of histidine, valine and tryptophan (0.95, 1.74 and 0.50) respectively in CL diet, the values of EAAs in SP were statistically higher than both reference and CL test diets ($p < 0.05$) although the difference between SP and CL in valine and phenylalanine contents was not significant, ($p > 0.05$).

As seen in Table 3.3, PUFA (35.47 - 44.27%) is the most abundant fatty acid fraction in all the diets, while C183n6 was the least (0.44- 0.45 %) of total fatty acids. CL diet was significantly better ($p < 0.05$) than reference and SP test diet in PUFA, C182n6c, C183n3, C184n3 and C203n3. *Spirulina* test diet was better ($p < 0.05$) than other diets in SFA, C183n4 and C226n3 (DHA), while reference diet exhibited significantly better MUFA C203n6 and C205n3 values than all other diets in this study ($p < 0.05$).

4.1.3 Growth performance

There was no mortality recorded during the experimental period, and all fish exhibited good growth. There was a statistical difference at $P < 0.05$, among groups, as shown in Table 4.1 in the mean weight gain, FCR, SGR, PI and PER. *Spirulina* was significantly better than the reference diet in weight gain, SGR, protein intake with a matching lower FCR. However, apart from protein intake, there was no observable statistical difference between the two test diets in the said parameters. *Chlorella*, on the other hand, exhibited significantly higher protein efficiency ratio (PER) than both *Spirulina* and the reference diet.

Table 4.1: Growth performance of *C. gariepinus* juveniles Fed FM SP or CL diets

	REFDT	SDT	CDT	P Value
Initial weight	57.99±0.06^{a}	58.02±0.01^{a}	57.99±0.06^{a}	0.902
Final weight	158.16±0.07^{b}	164.49±0.07^{a}	164.27±0.09^{a}	0.001
Weight Gain	100.17±0.07^{b}	106.47±0.09^{a}	106.28±0.06^{a}	0.001
FC	80.93±0.02^{a}	80.92±0.05^{a}	80.92±0.04^{a}	0.946
FCR	0.81±0.01^{a}	0.76±0.02^{b}	0.76±0.01^{b}	0.001
SGR	2.87±0.01^{b}	2.98±0.01^{a}	2.97±0.01^{a}	0.001
PI	32.17±0.02^{b}	33.18±0.03^{a}	31.86±0.02^{c}	0.001
PER	3.12±0.04^{c}	3.21±0.01^{b}	3.34±0.03^{a}	0.001
%Survival	100±0.0^{a}	100±0.0^{a}	100±0.0^{a}	-

Results are triplicate means the value of ten fishes. Mean values in the same row with different subscript are significant $P < 0.05$ S ADC: (Apparent Digestibility Coefficient), FC: (Feed Consumed), FCR: (Feed Conversion Ratio), SGR: (Specific Growth Rate), PI: (Protein Intake), PI: (Protein Intake and PER: Protein Efficiency Ratio); REFDT: **(**Reference Diet; SDT & CDT: *Spirulina* and *Chlorella* diets).

4.1.4 Digestibility of nutrients and energy of ingredients (*Spirulina* and *Chlorella*)

The ADC values of ingredients for protein, dry matter (DM), fat, ash, nitrogen-free extract (NFE), fibre and energy were different between the reference and the two test diets. The nutrients in reference and both test ingredients were digested to the highest extent in the catfish. The ADCs of 91.97%, 99.16%, 48.75%,56.53%,91.38% and 94.66% of *Spirulina* was significantly higher ($p<0.05$) than 80.62%, 97.71%, 92.77%,44.34%,39.71%,65.47% and 86.04% of FM for DM, protein, ash, crude fibre, NFE and gross energy respectively. Except for DM, NFE and GE there was no statistical difference between the SP and CL. *Chlorella*, on the other hand, achieved statistical significance over FM in ADC lipid and all the nutrients (Table 4.2).

Table 4.2: Apparent Digestibility Coefficients (ADC) of nutrients and energy (Kcal 100 g^{-1}) in the test ingredients Fed to *C. gariepinus* juveniles

Nutrients	Fishmeal	*Spirulina*	*Chlorella*	P Value
Dry matter	80.62±0.01 ^{c}	91.97±0.16 ^{a}	90.53±0.12 ^{b}	0.001
Crude Protein	97.71±0.05^{b}	99.16±0.04 ^{a}	99.17±0.02 ^{a}	0.001
Crude Lipid	92.77±0.64^{b}	96.56±0.09 ^{a}	96.71±0.09 ^{a}	0.001
Crude Ash	44.34±0.02 ^{b}	48.75±0.09 ^{a}	48.57±0.05 ^{a}	0.347
Crude fibre	39.71±4.17 ^{b}	56.53±0.70 ^{a}	56.06±0.93 ^{a}	0.005
NFE	65.47±0.05 ^{c}	91.38±0.18 ^{a}	87.72±1.36 ^{b}	0.001
Gross Energy	86.04±0.12^{c}	94.66±0.10 ^{a}	93.20±0.46 ^{b}	0.001

Mean values with different superscript across the rows were significantly differenced $p < 0.05$. N = 3 ± standard error (SE).

4.1.5 Digestibility of amino acids of ingredients (fishmeal, *Spirulina* and *Chlorella*)

The ADCs of all EAAs amongst both microalgae ingredients achieved statistical significance ($p < 0.05$) except for those of threonine, methionine, leucine and phenylalanine (Table 4.3). The ADC of most individual EAAs were significantly higher in CL than FM and SP. SP lysine content was however significantly ($p < 0.05$) more digestible than that of CL.

Table 4.3: Apparent Digestibility Coefficients (ADC) of amino acids in the test ingredients Fed to *C. gariepinus* juveniles

Nutrients	Fishmeal	*Spirulina*	*Chlorella*	P Value
Histidine*	97.50±0.07 c	98.91±0.01 b	99.04 ±0.01 a	0.001
Arginine*	98.52±0.01c	99.32±0.02 b	99.41±0.01 a	0.005
Threonine*	97.67±0.04 b	98.75±0.02 a	98.80±0.02 a	0.158
Valine*	96.35±0.02 c	98.30±0.02 b	98.50±0.01 a	0.000
Methionine*	99.20±0.01 b	99.56±0.03 a	99.51±0.01 a	0.002
Lysine*	98.59±0.01 c	99.34±0.04 a	99.05±0.01 b	0.002
Isoleucine*	96.75±0.02 c	98.57±0.01 b	98.63±0.01 a	0.002
Leucine*	96.85±0.02 b	98.93±0.06 a	98.93±0.02a	0.959
Phenylalanine*	97.13±0.02 b	98.78±0.07 a	98.83±0.02 a	0.603
Tryptophan*	98.27±0.03 c	99.45±0.02 b	99.79±0.01 a	0.000

Mean values with different superscript across the rows were significantly differenced $p < 0.05$. N = 3 ± standard error (SE).

4.1.6 Digestibility of fatty acids of FM and test ingredients (*Spirulina* and *Chlorella*)

The ADCs of all the FA fractions among FM and both test microalgae achieved statistical significance except for C183n3, C184n3, C203n3, DHA and ∑n-3 (Table 3.10) The ADC % values of total SFA, MUFA, PUFA and C183n4 were significantly higher in SP (99.66, 97.50, 99.36 and 99.60) than in CL (98.75, 98.75, 99.04 and 98.46). The ADC values of ∑n-6 (comprising C182n6t, C182n6C, C183n6 and C203n6) in CL were however significantly better than that of SP (99.31) and 99.07) individually. There was no remarkable difference $p > 0.05$ between CL and SP in other fractions of the ∑n3 (C183n3, C184n3, C203n3, EPA and DHA) groups. Except for C182n6t, both SP and CL were however significantly better in all the tested FA groups (Table 4.4).

Table 4.4: Apparent digestibility coefficients (ADC) of fatty acids of the test ingredients diets Fed to *C. gariepinus* juveniles

Fatty Acids	Fishmeal	*Spirulina*	*Chlorella*	P value
SFA	97.59± 0.00 c	99.66±0.02 a	98.75±0.08 b	0.000
MUFA	96.08 ± 0.06 c	97.50±0.03 a	96.70±0.01 b	0.000
PUFA	97.51±0.04 b	99.36±0.01 a	99.04±0.01 b	0.000
C182n6t	99.06±0.06 ab	98.83. ± 0.01b	99.46 ± 0.02 a	0.03
C182n6c	98.40± 0.01 c	99.37 ± 0.01 b	99.68 ± 0.01 a	0.00
C183n4	96.79 ± 0.19 c	99.60±0.01 a	98.51±0.02 b	0.000
C183n6	97.67± 0.13 b	98.46 ± 0.02 b	98.77 ± 0.01 a	0.000
C183n3	99.83 ± 0.00 c	99.89 ± 0.01 a	99.91 ± 0.01 a	0.101
C184n3	99.68 ± 0.00 b	99.81 ± 0.02 a	99.85 ± 0.01 a	0.132
C203n6	97.41± 0.06 b	98.18± 0.01 b	98.27 ± 0.02 a	0.012
C203n3	99.68± 0.01b	99.83 ± 0.01a	99.85 ± 0.01 a	0.251
C205n3 EPA	98.45± 0.06b	99.07±0.02a	99.13±0.01 a	0.021
C226n3 DHA	98.79± 0.17 c	99.64±0.02 a	99.68±0.01 a	0.168
∑n-6	98.37±0.02 c	99.31±0.02 b	99.66±0.01 a	0.000
∑n-3	99.34±0.02 b	99.86±0.01 a	99.88±0.01a	0.067
n3/n6 PUFA ratio	2.82±0.01b	0.34±0.01b	1.39±0.02a	.000

Mean values with different superscript (a-c) across the rows were significantly differenced $p < 0.05$. N = 3 ± standard error (SE). FA: Fatty acids; TFA: Total fatty acids; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, SDT: *Spirulina* diet; CDT: *Chlorella* diet.

4.1.7 Digestibility of nutrients and energy in reference and test diets

The test diets achieved statistical superiority of $P < 0.05$ in the ADC of energy and nutrients by the *C. gariepinus* juveniles in the present study. *Spirulina* diet was higher to reference diet and *Chlorella* in ADC of lipids, dry matter, fibre, NFE and energy. Whereas, *Chlorella* diet exhibited higher protein, lipid and ash digestibility. There was no significant difference $p > 0.05$ in the *C. gariepinus* ability to digest protein of *Chlorella* and *Spirulina* diets (Table 4.5).

4.1.8 Amino acids digestibility of reference and test diets

As seen in table 4.6, all the 18 amino acids were well digested by the experimental fishes. Both test diets were significantly better $p < 0.05$ than the reference diets in terms the entire amino acid digestibility by the experimental fishes. The ADC of eight individual essential amino acids (EAAs) namely histidine, arginine, threonine, valine, isoleucine, leucine phenylalanine, and tryptophan were higher $p < 0.05$ in the *Chlorella* diet when compared to the reference and *Spirulina*. Other non-essential amino acids (NEAAs) in which *Chlorella* also has a comparative advantage over the reference diet, were serine and glycine. However, besides arginine, alanine, valine and tryptophan, there was no statistical difference between *Chlorella* and *Spirulina* in the other three aforesaid amino acids.

Spirulina test diet was significantly better than reference diet in the availability of methionine, lysine, EAAs, aspartic acid, glutamic acid, alanine, proline, and tyrosine NEAAs. Apart from aspartic acid, glutamic acid tyrosine and lysine, there was no significant difference $p < 0.05$ between the availability of all the said amino acids of *Spirulina* and *Chlorella*. Tryptophan was observed to be the most digestible EAA compound (94.47 – 99.79%) followed by methionine (99.20- 99.56, while valine has the least digestibility value of 96.35-98.58.

Table 4.5: Apparent digestibility coefficients (ADC) of nutrients in the experimental diets Fed to *C. gariepinus* juveniles

Nutrients	REFDT	SDT	CDT	P value
$ADC_{Protein}$	97.71±0.06 ^{b}	98.64±0.02 ^{a}	98.66±0.02 ^{a}	0.001
ADC_{Lipid}	92.77±0.64 ^{b}	96.51±0.08 ^{a}	96.67±0.12 ^{a}	0.001
$ADC_{Dry\ matter}$	80.62±0.01 ^{c}	91.82±0.16^{a}	90.42± 0.11^{b}	0.001
ADC_{Ash}	44.34±0.02 ^{b}	48.72±0.10 ^{a}	48.52±0.10 ^{a}	0.001
ADC_{Fibre}	39.71±4.17 ^{b}	56.53±0.70 ^{a}	56.05±0.93 ^{a}	0.005
ADC_{NFE}	65.47±0.05 ^{c}	88.30±0.15 ^{a}	84.72±1.17 ^{b}	0.001
ADC_{Energy}	86.04±0.12 ^{c}	94.66±0.10 ^{a}	93.20±0.46 ^{b}	0.001

Mean values with different superscript (a, b & c) across the rows were significantly difference $p < 0.05$. N = 3 ± standard error (SE). ADC: Apparent Digestibility Coefficient, REFDT: Reference diet; SDT &CDT: *Spirulina* and *Chlorella* diets

Table 4.6: Apparent digestibility of amino acids in the experimental diets Fed to *C. gariepinus* juveniles

Compounds	REFDT	SDT	CDT	P Value
Hydroxyproline	88.13±0.02^{a}	76.43±2.41^{c}	81.96±1.07^{b}	0.05
Aspartic Acid	97.53±0.09 ^{c}	98.63±0.07 ^{a}	98.34±0.02 ^{b}	0.001
Serine	96.58±0.03 ^{b}	97.95±0.12 ^{a}	98.11±0.05 ^{a}	0.001
Glutamic Acid	97.27±0.03 ^{c}	98.85±0.06 ^{a}	98.53±0.03 ^{b}	0.001
Glycine	95.53±0.07 ^{b}	97.75±0.12 ^{a}	97.82±0.07 ^{a}	0.001
Histidine*	97.50±0.07 ^{b}	98.92±0.06^{a}	99.04±0.04 ^{a}	0.001
Arginine*	98.52±0.01^{c}	99.33±0.04 ^{b}	99.41±0.01 ^{a}	0.001
Threonine*	97.67±0.04 ^{b}	98.75±0.07^{a}	98.80±0.01 ^{a}	0.001
Alanine	92.63±0.07 ^{c}	97.20±0.15 ^{b}	96.80±0.03 ^{a}	0.001
Proline	93.81±0.03 ^{b}	97.40±0.16 ^{a}	97.23±0.01 ^{a}	0.001
Tyrosine	98.58±0.07^{c}	98.64±0.08 ^{a}	97.92±0.01 ^{b}	0.001
Valine*	96.35±0.02 ^{c}	98.30±0.12 ^{b}	98.58±0.02 ^{a}	0.001
Methionine*	99.20±0.01 ^{b}	99.56±0.08 ^{a}	99.52±0.02 ^{a}	0.001
Lysine*	98.59±0.01 ^{c}	99.34±0.04 ^{a}	99.05±0.01 ^{b}	0.001
Isoleucine*	96.75±0.02 ^{b}	98.56±0.08 ^{a}	98.63±0.01 ^{a}	0.001
Leucine*	96.85±0.02 ^{b}	98.92±0.06 ^{a}	98.93±0.01 ^{a}	0.001
Phenylalanine*	97.13±0.02 ^{b}	98.78±0.07 ^{a}	98.83±0.02 ^{a}	0.001
Tryptophan*	98.27±0.03 ^{c}	99.45±0.02 ^{b}	99.79±0.01 ^{a}	0.001

Values are means of triplicate samples ± Standard Error of Mean (SE). Mean values with different superscript (a, b & c) across the rows was significantly difference $p < 0.05$ * Essential Amino acids; REFDT: Reference diet; SDT &CDT: *Spirulina* and *Chlorella* diets.

4.1.9 Digestibility of fatty acids in test and reference diets

The ADCs of FAs in all the experimental diets were above 90% with both test diets better than the reference diets in the ADCs of all FA segments (Table 4.7). The most digestible fatty acid fraction was C183n3 (99.83- 99.91) and MUFA with the digestibility

value range of 96.08 - 97.41% was the least digestible. Except for monounsaturated fatty acid (MUFA), total saturated fatty acid (SFA) and C183n4 which was significantly highest $p < 0.05$ in *Spirulina*; ADCs of the fractions of fatty acid were higher significantly, $p < 0.05$, in *Chlorella* diets than reference and *Spirulina* diets. The ADCs of total PUFA, ∑ n-6 and n-3 PUFA (C182n6t, C182n6C, C183n6, C183n3, C184n3, C203n6, C203n3), EPA (C205n3) and DHA (C226n3) were greater in *Chlorella* diet than reference. However, apart from C182n6t, C182n6C and C183n3, the difference between the ADC of *Chlorella* and *Spirulina* diets in this study was not statistically significant, $p > 0.05$.

Table 4.7: Apparent Digestibility Coefficients (ADC) of Fatty Acids in the experimental diets Fed to *C. gariepinus* juveniles

Fatty Acids	RFDT	SDT	CTD	P value
SFA	97.59± 0.00 ^{c}	98.98 ± 0.06 ^{a}	98.84 ± 0.01 ^{b}	.001
MUFA	96.08 ± 0.06 ^{c}	97.41 ± 0.15 ^{a}	96.74 ± 0.04^{b}	.001
PUFA	97.51±0.04 ^{b}	98.92±0.02 ^{b}	99.01 ± 0.01 ^{a}	.001
C182n6t	99.06± 0.06 ^{ab}	98.83 ± 0.21 ^{a}	99.46 ± 0.02^{a}	.003
C182n6c	98.40± 0.01 ^{c}	99.33 ± 0.04 ^{b}	99.67 ± 0.01 ^{a}	.001
C183n4	96.79 ± 0.19 ^{c}	99.60 ± 0.01 ^{a}	98.51 ± 0.13 ^{b}	.001
C183n6	97.67± 0.13 ^{b}	98.46 ± 0.02 ^{b}	98.77 ± 0.05 ^{a}	.001
C183n3	99.83 ± 0.00 ^{c}	99.90 ± 0.01 ^{b}	99.91 ± 0.00 ^{a}	.001
C184n3	99.68 ± 0.00 ^{b}	99.81 ± 0.02 ^{a}	99.85 ± 0.01 ^{a}	.001
C203n6	97.41± 0.06 ^{b}	98.18 ± 0.11 ^{a}	98.27 ± 0.07 ^{a}	.001
C203n3	99.68± 0.01^{b}	99.83±0.01 ^{a}	99.85 ± 0.00 ^{a}	.001
C205n3	98.45± 0.06^{b}	99.07± 0.07 ^{a}	99.13± 0.02 ^{a}	.001
C226n3	98.79± 0.17 ^{c}	99.64 ± 0.02 ^{a}	99.68± 0.00 ^{a}	.001
∑n-6	98.37±0.02 ^{c}	99.26±0.05 ^{b}	99.65±0.02 ^{a}	.001
∑n-3	99.34±0.02 ^{b}	99.85±0.02^{a}	99.87±0.02 ^{a}	.001
n3/n6 ratio	2.82±0.01^{b}	2.94 ±0.02^{a}	1.49 ±0.03 ^{c}	.001

Mean values with different superscript (a, b & c) across the rows were significantly differenced $p < 0.05$. N = 3 ± standard error (SE). FA: Fatty acids; TFA: Total fatty acids; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, REFDT: Reference diet; SDT &CDT: *Spirulina* and *Chlorella* diets

4.2 Effects of partial replacements of fishmeal with *Spirulina platensis* and *Chlorella vulgaris* on growth performance and body composition of African catfish (*Clarias gariepinus*) fingerlings

4.2.1 Proximate compositions of growth experiment diets

Table 3.4 shows formulations and compositions of the different investigation of diets in this study. Crude protein of the experimental diets (g100 g^{-1}) iso-nitrogenous at ≈ 45% and iso-caloric ≈ 10% and iso-energetic at 364.45 Kcal100 g100 g^{-1}. The ash content is 10.14-10.47, moisture 9.02- 9.47 7.3, fibre 2.51-2.86, NFE 20.58 -22.28.

4.2.2 Amino acids compositions of experimental diets

All the 18 amino acids namely: hydroxyproline, methionine, lysine, threonine, alanine, proline, aspartic acid, serine, glutamic acid, glycine, histidine, arginine, tyrosine, valine isoleucine, leucine, phenylalanine and tryptophan that was analysed for were present in varying amounts in all the diet (Table 3.5). Of all the ten essential amino acids, SP50% was considerably greater (P <0.05) than all diets in phenylalanine and arginine, threonine, (except for SP12.5%) histidine and methionine (except for SP50%). SP25% was significantly greater (P <0.05) in valine (except for SP12.5% and SP50%) and lysine (except CL12.5%) while SP12.5% was significantly better (P <0.05) in tryptophan and isoleucine than all other diets (except for SP25% and SP50%). SP50% was also significantly higher in leucine content.

4.2.3 Fatty acids compositions of experimental diets

Significant differences (P <0.05) were observed in total saturated fatty acid (4:0, 6:0, 8:0, 10:0, 11:0,12:0, 13:0,14:0, 15:0, 16:0, 18:0, 20:0, 22:0,and 24:0), total monosaturated (14:1,16:1,15:1,16:1, 18:1n9c, 18:1n7, 20:1n9, C22:1n9 and 24:1n9) and total n-6 (18:2n6t, 18:2n6c, 18:3n6) and n-3 (18:3n3, 18:4n3), and overall n-3(20:3n3, 20:5n3 and 22:6n3) and n-6 (20:3n6) LC-PUFA among dietary treatments (Table 3.6).

The CL12.5% diet had significantly higher total fatty acids (TFA) than control and other treatments, while SP50% had significantly higher C18:2n6c (13.70%) and saturated fatty acids (SFA) majority of which was made up of C16:0. Total monounsaturated predominated in SP12-5% (24.33%), a substantial amount of which was made up of 18:1n9c (12.6%) as found in SP25%.

Both linolenic and docosahexaenoic (18:3n3 and 22:6n3DPA) were higher in CL25 % while (20:5n3) eicosapentaenoic acid (EPA) was higher in CL75 % diet.

4.2.4 Growth parameters of fingerlings Fed experimental diets

There was no significant mean differences ($P > 0.05$) amongst *Spirulina* (SP) and *Chlorella* (CL) treatment groups of *C. gariepinus* in initial weight (IW), feed intake (FDCOM) and survival of fingerlings used for this study at F (1, 20) = 0.40, p = 0.54, F (1, 20) = 7.06, p = 0.13, F (1, 20) = 0.048, p = 0.829, respectively. There were statistical significant mean differences ($P < 0.05$) between the two groups in final fish weight (FW) F (1, 20) = 563.64, p < 0.001, weight gain F (1, 20) = 340.01, p < 0.001, FCR F (1, 20) = 875.84, p < 0.001, PER F (1, 20) = 5216.45, p < 0.001, PRTIN F (1, 20) = 5322.32, p < 0.001, SGR F (1, 20) = 196.00, p < 0.001, PPV F (1, 20) = 38.12, p < 0.001, RGR F (1, 20) = 50.31, p < 0.001 and Fulton's condition factors (K) F (1, 20) = 61.07, p < 0.001, *Chlorella* exhibited higher comparative advantage in all aforementioned significant parameters with a corresponding lower FCR than *Spirulina*.

The effect of dietary levels shows there was no significant mean difference in IW, FDCOM and survival in fingerlings used for this study F (4, 20) = 0.71, P = 0.598, F (4, 20) = 0.16, P = 0.957 and F (4, 20) = 1.74, P = 0.181 respectively. There was a significant mean difference of dietary levels in final fish weight (FW) F (4, 20) = 48.11, p < 0.001, weight gain F (4, 20) = 26.99, p < 0.001, FCR F (4, 20) = 114.00, p < 0.001, PER F (4,

20) = 489.95, p < 0.001, PRTIN F (4, 20) = 564.09, p < 0.001, SGR F (4, 20) = 19.13, p < 0.001, PPV F (4, 20) = 32763.25, p < 0.001, RGR F (4, 20) = 3.78, p = 0.019 and Fulton's condition factors (K) F (4, 20) = 11.39, p < 0.001.

As shown in Table 4.8, there was no significant interaction (p > 0.05) between algae group of *C. gariepinus* and dietary levels in IW, FDCOM and survival (in fingerlings); indicating that the pattern of differences in all pairs dietary levels for IW, FDCOM and survival is the same for *Spirulina* and *Chlorella*. All the pairs of dietary levels are not significantly different for both *Spirulina* and *Chlorella* in these parameters. There was a significant interaction (p < 0.05) between algae group and dietary levels in FW, WG, FCR, PER, PRTIN, SGR, PPV, RGR and K, thus indicating that the pattern of differences between the pairs of dietary levels is not the same for *Spirulina* and *Chlorella***.** For FW and WG the pattern of differences for the pairs was similar with control having the lowest mean and 75% having the highest mean in both *Spirulina* and *Chlorella*, but in *Chlorella* the means of 50% and 75% were much higher than in *Spirulina*. FCR control has the highest mean for both *Spirulina* and *Chlorella*, however, in *Spirulina* 50% has the lowest mean, while in the *Chlorella* group, 75% has the lowest mean. For PER control has the lowest mean for both *Spirulina* and *Chlorella*, SP50% and CL75% had the highest means for *Spirulina* and *Chlorella* respectively. For SGR 75% inclusion has the highest mean for both *Spirulina* and *Chlorella*. In contrast to *Spirulina* where control had the lowest mean value, 12.5% level of *Chlorella* had the lowest SGR mean. For PPV in *Spirulina* 75% has the lowest mean and 12.5% has the highest mean whereas *Chlorella* 25% had the lowest mean and 50% had the highest mean value. For RGR all the pairs of dietary levels for *Chlorella* are significantly different but in *Spirulina* 50% and 75% are not significantly different. Nevertheless, control had the lowest mean in both *Spirulina* and *Chlorella* and 75% inclusion level had the highest mean for both *Spirulina* and *Chlorella*. For *Spirulina* K factor, there were no significant differences between 25%,

50% and 75%, but in *Chlorella* all pairs were significantly different. The control for both *Spirulina* and *Chlorella* had the lowest mean for K factor and 75% had the highest mean values for both algae.

The regression coefficient of different percentage increments of both *Spirulina* and *Chlorella* as fishmeal replacement was significant ($P < 0.05$). The R^2 value of 0.9468 and 0.9776 in *Spirulina* and *Chlorella* (Figure 4.1 & 4.2) indicated that there is a positive correlation between both *Spirulina* and *Chlorella* and weight gain as the experimental fish exhibited good growth. Optimum replacement of FM (as calculated from the polynomial curve equation) with *Spirulina* is about 68.5 g100 g^{-1} and *Chlorella* 69.41 g 100 g^{-1} (68.5% and 69.41%).

4.2.5 Proximate composition of the experimental fish carcass

Proximate composition of the fish carcass (Table 4.9) revealed a significant difference ($P < 0.05$): protein (F (9,20) = 21.27, $P = 0.000$,), lipid (F (9,20) = 1650.19, $P = 0.000$), ash (F (9,20) = 688.64, $P = 0.000$), moisture (F (9,20) = 76.77, $P = 0.000$), NFE (F (9,20) = 26.41, $P = 0.000$) and gross energy contents (F (9,20) = 659.90, P = 0.000), of all experimental fish groups.

The mean protein value (M = 61.97, SE = 0 .10) recorded for SP12.5 % was significantly different ($P < 0.05$) with all other experimental fish groups except for SP25 %. Flesh lipid (M = 10.52, SE = 0.08) and gross energy (M = 473.96, SE = 1.65) were significantly higher ($P < 0.05$) in SP75 % whereas the initial fish demonstrated significantly ($P < 0.05$) higher ash (M = 20.95, SE = 0.04), moisture (M = 5.00 ± 0.03), and NFE (M = 12.04, SE = 0.08) than all the other fish group in this study.

Table 4.8: Two-way ANOVA for the effect of algae (SP&CL) and dietary inclusion levels on growth parameters of *C. gariepinus* fingerlings for 56 days

TRT	IW	FW	WG	FDCOM	FCR	PER	SGR	PPV	RGR	Futon-K	%SURVIVAL
Algae type											
Spirulina	7.86±0.2	21.67±0.0 ^{A}	13.81±0.02^{A}	17.82±0.02	1.26±0.01^{B}	1.72±0.01^{A}	3.04±0.01^{A}	60.74±0.06^{A}	175.76±0.56^{A}	1.59±0.01^{A}	94.67±2.16
Chlorella	7.84±0.02	22.06±0.01^{B}	14.22±0.02^{B}	17.77±0.02	1.18±0.01^{A}	1.93±0.01^{B}	3.05±0.01^{B}	61.23±0.06^{B}	181.39±0.56^{B}	1.67±0.01^{B}	94.00±2.16
Diet level											
Control	7.82±0.03	19.29±0.02^{A}	11.47±0.02^{A}	17.77±0.04	1.55±0.01^{E}	1.35±0.01^{A}	2.92±0.01^{A}	58.19±0.09^{B}	146.63±0.89^{A}	1.39±0.01^{A}	86.67±3.42
12.5%	7.87±0.03	20.17±0.02^{B}	12.30±0.02^{B}	17.79±0.04	1.37±0.01^{D}	1.53±0.01^{B}	2.97±0.01^{B}	72.00±0.09^{E}	156.20±0.89^{B}	1.54±0.01^{B}	96.67±3.42
25%	7.85±0.03	22.46±0.02^{C}	14.62±0.02^{C}	17.81±0.04	1.14±0.01^{C}	1.95±0.01^{C}	3.08±0.01^{C}	59.29±0.09^{C}	186.27±0.89^{C}	1.66±0.01^{C}	95.00±3.42
50%	7.88±0.03	23.59±0.02^{D}	15.71±0.02^{D}	17.80±0.04	1.00±0.01^{A}	2.18±0.01^{E}	3.12±0.01^{D}	61.18±0.09^{D}	199.43±0.89^{D}	1.77±0.01^{D}	95.00±3.42
75%	7.83±0.03	23.83±0.02^{E}	16.00±0.02^{E}	17.80±0.04	1.04±0.01^{B}	2.12±0.01^{D}	3.14±0.01^{E}	54.27±0.09^{A}	204.35±0.89^{E}	1.81±0.01^{E}	98.33±3.42
Algae × dietary level											
SP × Ctrl	7.82±0.04	19.29±0.03^{A}	11.47±0.04^{A}	17.77±0.05	1.55±0.01^{E}	1.35±0.01^{A}	2.92±0.01^{A}	58.19±0.13^{C}	146.63±1.25^{A}	1.39±0.02^{A}	86.67±4.83
SP×12.5%	7.88±0.04	19.91±0.03^{B}	12.04±0.04^{B}	17.83±0.05	1.40±0.01^{D}	1.41±0.01^{B}	2.95±0.01^{B}	83.61±0.13^{E}	152.90±1.25^{B}	1.50±0.02^{B}	96.67±4.83
SP× 25%	7.88±0.04	22.33±0.03^{C}	14.45±0.04^{C}	17.85±0.05	1.17±0.01^{C}	1.82±0.01^{C}	3.07±0.01^{C}	78.84±0.13^{D}	183.53±1.25^{C}	1.66±0.02^{C}	96.67±4.83
SP× 50%	7.87±0.04	23.30±0.03^{D}	15.43±0.04^{D}	17.84±0.05	1.05±0.01^{A}	2.09±0.01^{E}	3.11±0.01^{D}	46.80±0.13^{B}	196.07±1.25^{D}	1.69±0.02^{C}	93.33±4.83
SP× 75%	7.85±0.04	23.52±0.03^{E}	15.67±0.04^{E}	17.81±0.05	1.14±0.01^{B}	1.92±0.01^{D}	3.12±0.01^{E}	36.24±0.13^{A}	199.69±1.25^{E}	1.73±0.02^{C}	100.00±4.83
CL ×Ctrl	7.82±0.04	19.29±0.03^{A}	11.47±0.04^{A}	17.77±0.05	1.55±0.01^{E}	1.35±0.01^{A}	2.92±0.01^{B}	58.19±0.13^{B}	146.63±1.25^{A}	1.39±0.02^{A}	86.67±4.83
CL×12.5%	7.87±0.04	20.42±0.03^{B}	12.55±0.04^{B}	17.74±0.05	1.34±0.01^{D}	1.65±0.01^{B}	2.98±0.01^{A}	60.39±0.13^{C}	159.50±1.25^{B}	1.58±0.02^{B}	96.67±4.83
CL × 25%	7.82±0.04	22.60±0.03^{C}	14.78±0.04^{C}	17.77±0.05	1.11±0.01^{C}	2.07±0.01^{C}	3.08±0.01^{C}	39.73±0.13^{A}	189.01±1.25^{C}	1.67±0.02^{C}	96.67±4.83
CL × 50%	7.89±0.04	23.88±0.03^{D}	15.99±0.04^{D}	17.76±0.05	0.95±0.01^{B}	2.27±0.01^{D}	3.14±0.01^{D}	75.55±0.13^{E}	202.78±1.25^{D}	1.84±0.02^{D}	96.67±4.83
CL × 75%	7.81±0.04	24.14±0.03^{E}	16.32±0.04^{E}	17.79±0.05	0.93±0.01^{A}	2.33±0.01^{E}	3.15±0.01^{E}	72.31±0.13^{D}	209.02±1.25^{E}	1.89±0.02^{D}	98.33±3.42

Mean values (n = 30) in a column under each category of parameter bearing different superscript (upper case) vary significantly ($P < 0.05$), SGR: Specific Growth Rate; FCR: Feed Conversion Ratio; RGR: Relative Growth Rate &PER: Protein Efficiency Ratio; K: Fulton condition factor; PPV: Protein Productive Value; FODCOM: Feed consume.

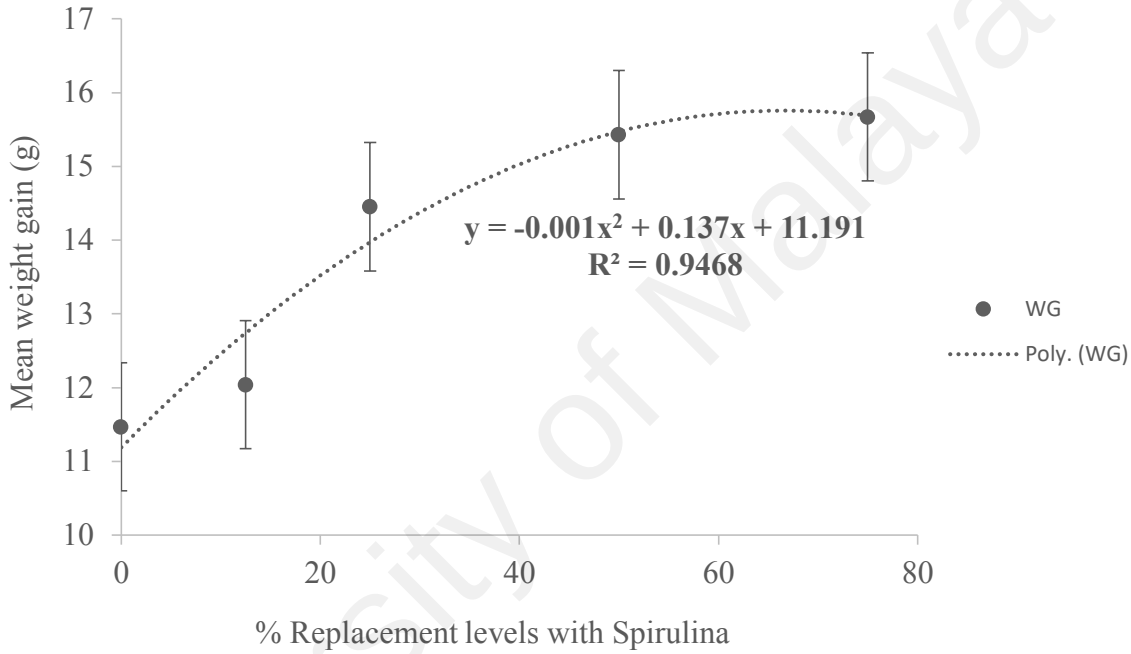


Figure 4.1: Mean weight gain of *C. gariepinus* fingerlings Fed graded levels of *Spirulina* for 56 days. The Optimum level occurs is 68.5%.

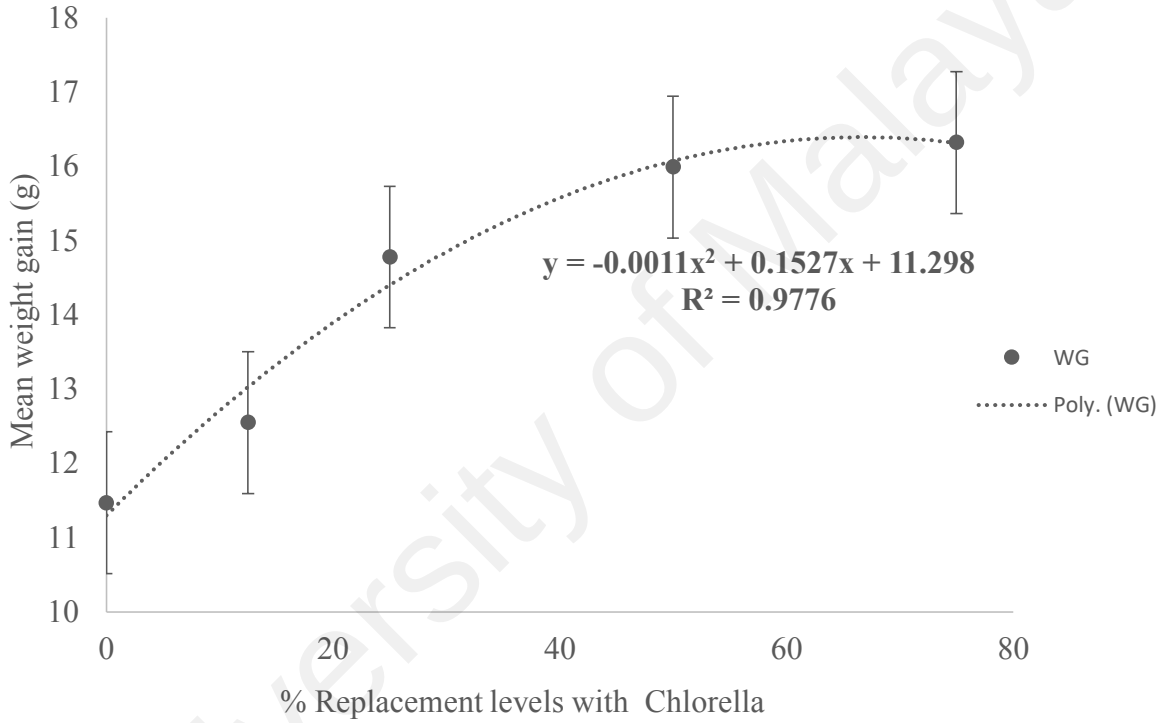


Figure 4.2: Mean weight gain of *C. gariepinus* fingerlings Fed graded levels of *Chlorella* for 56 days. The optimum level occurs is 69.4%

Table 4.9: Proximate composition of the whole body of *C. gariepinus* Fed *Spirulina* and *Chlorella* diets

	Initial	Control	SP12.5%	SP25%	SP50%	SP75%	CL12.5%	CL25%	CL50%	CL75%
CP	54.85±0.09 ^{g}	59.79±0.00 ^{d}	61.97±0.10 ^{a}	61.10±0.40 ^{b}	58.30±0.25 ^{e}	57.81±0.40 ^{f}	59.44±1.07 ^{d}	57.68±0.17 ^{f}	60.17±0.24 ^{c}	59.93±0.57 ^{c}
CL	7.16±0.00 ^{e}	10.21±0.03 ^{a}	7.53±0.01^{d}	9.10±0.00 ^{c}	9.61±0.03^{b}	10.52±0.08 ^{a}	9.78±0.15 ^{b}	9.63±0.11^{b}	7.16±0.00 ^{e}	9.01±0.03 ^{c}
Ash	20.95±0.04 ^{a}	17.71±0.11 ^{e}	18.39±0.10 ^{c}	18.82±0.00 ^{b}	17.62±0.01^{e}	16.08±0.05 ^{g}	16.78±0.06 ^{f}	18.04±0.00 ^{d}	16.93±0.06 ^{f}	16.54±0.01 ^{g}
Moist	5.00±0.03 ^{a}	2.83±0.01 ^{e}	4.04±0.00 ^{b}	3.99±0.01 ^{c}	3.92±0.01 ^{c}	3.61±0.19 ^{d}	3.47±0.00 ^{d}	3.63±0.03 ^{d}	4.07±0.03 ^{b}	3.71±0.03 ^{d}
NFE	12.04±0.08 ^{a}	9.46±0.08 ^{d}	8.07±0.01 ^{f}	6.99±0.38 ^{g}	10.55±0.22^{d}	11.98±0.34 ^{b}	10.53±0.98 ^{d}	11.02±0.03^{c}	11.67±0.14 ^{b}	10.81±0.50 ^{c}
GE	425.71±0.20 ^{g}	472.17±0.56 ^{b}	453.57±0.49 ^{f}	459.17±0.72 ^{e}	462.41±0.26 ^{d}	473.96±1.65 ^{a}	470.38±0.9^{c}	460.98±0.20 ^{d}	454.30±0.75 ^{f}	466.99±0.93^{d}

Values are means of triplicate groups of 3 fish per replicate. Mean values with different subscript within the same row (a - g) are significantly different at ($P<0.05$). CP: Crude protein; CL: Crude lipid; NFE> Nitrogen Free Extract; GE: Gross energy

4.3 Effects of *Spirulina* and *Chlorella* on growth and haemato-immunological response of African catfish to pathogenic *Aeromonas hydrophila.*

4.3.1 Fish growth performance prior *Aeromonas hydrophila* challenge compositions of growth experiment diets.

Final fish weights were taken on day 112 (16 weeks). Table 4.10 shows the growth parameters of experimental fish prior to bacterial challenge.

4.3.1.1 Treatment (algae type) effect

There were no statistical differences between *Spirulina* and *Chlorella* in terms of all measured growth parameters namely: fish final weight, weight gain, feed consumption, FCR, SGR, PER, PI, RGR and K-factor.

Dietary inclusion level (control. 50% and 75%) effect as seen in Table 4.10, there were significant mean differences of final weight between the dietary levels Post hoc indicate mean of final weight, body weight gains, SGR, RGR and Fulton's K-factor in 50% and 75% meal were significantly higher than in control, but no significant difference between 50% and 75% meals.

4.3.1.2 The interaction term for algae and diet level

The effects of both algae and their inclusion levels were, however, not significant ($P > 0.05$) on all the afore-mentioned growth parameters, indicating that, there were no significant interactions ($p > 0.05$) between algae type and dietary inclusion level for all the measured growth performance parameters.

Table 4.10: Two-way ANOVA for the effect of Algae (SP&CL) and dietary inclusion levels on growth parameters of *C. gariepinus* fishes Fed different graded levels of *Spirulina* and *Chlorella* for 16 weeks prior *A. hydrophila* challenge.

Treatment	FW	IW	WG	FC	FCR	SGR	PER	PI	RGR	K Factor
SP	272.64±0.18	41.85±0.07	230.79±0.16	121.70±4.39	0.53±0.02	2.27±0.01	5.29±0.16	44.04±1.59	551.50±1.09	1.83±0.01
CL	272.54±0.18	41.85±0.07	230.6±0.16	120.48±4.39	0.52±0.02	2.27±0.01	5.39±0.16	43.27±1.59	551.56±1.09	1.84±0.01
Diet level										
CTRL	267.13±0.22^{A}	41.85±0.08	225.28±0.20 ^{A}	128.43±5.38	0.57±0.02	2.25±0.01^{A}	4.95±0.19	46.38±1.94	538.91±1.34^{A}	1.75±0.02 ^{A}
50%	275.57±0.22 ^{B}	41.87±0.08	233.70±0.20 ^{B}	113.25±5.38	0.48±0.02	2.28±0.01^{B}	5.70±0.19	41.04±1.94	558.18±1.34^{B}	1.89±0.02 ^{B}
75%	275.07±0.22 ^{B}	41.84±0.08	233.23±0.20 ^{B}	121.60±5.38	0.52±0.02	2.28±0.01^{B}	5.36±0.19	43.54±1.94	557.50±1.34^{B}	1.87±-0.02 ^{B}
Algae ×dietary level										
SP × CTRL	267.33±0.31	41.84±0.11	225.49±0.28	128.43±7.60	0.57±0.03	2.25±0.01	4.95±0.27	46.38±2.75	538.91±1.89	1.75±0.02
SP × 50%	275.54±0.31	41.86±0.11	233.68±0.28	114.93±7.60	0.49±0.03	2.28±0.01	5.59±0.27	41.85±2.75	558.19±1.89	1.88±0.02
SP × 75%	275.05±0.31	41.84±0.11	233.21±0.28	121.75±7.60	0.52±0.03	2.28±0.01	5.32±0.27	43.90±2.75	557.40±1.89	1.87±0.02
CL × CTRL	266.92±0.31	41.85±0.11	225.07±0.28	128.43±7.60	0.57±0.03	2.25±0.01	4.95±0.27	46.38±2.75	538.91±1.89	1.75±0.02
CL × 50%	275.60±0.31	41.87±0.11	233.73±0.28	111.56±7.60	0.48±0.03	2.28±0.01	5.81±0.27	40.24±2.75	558.18±1.89	1.89±0.02
CL × 75%	275.09±0.31	41.83±0.11	233.26±0.28	121.44±7.60	0.52±0.03	2.28±0.01	5.40±0.27	43.19±2.75	557.59±1.89	1.87±0.02

Values are means of triplicates of ten fish/ tank. Mean values on the same row with different superscript are significantly different (P<0.05). FW: Final weight; IW: Initial weight; WG: Weight gain; FCR: Feed conversion ratio; SGR: Specific growth rate; PER: Protein efficiency ratio; PI: Protein intake; RGR: Relative growth rate.

4.3.2 Effect Algae types, inclusion levels and their interactions on haematological parameters pre and post *A. hydrophila Challenge*

Assumption for equality of variance using Leven's test: All the assumptions were met ($p > 0.05$), except for Post Hct, $p = 0.002$.There were no significant mean differences between the two types of algae (SP and CL) in pre- *A. hydrophila* challenge values of Hgb at $p = 0.46$, Hct= $p = 0.91$, RBC, $P = 0.63$, MCV $p = 0.61$, MCH $p = 0.63$, MCHC $p = 0.23$; RDW, $p = 0.77$ and platelets, $p = 0.64$. There was however a significant difference of pre-WBC for algae type, $F (1, 12) = 16.95$, $p=0.001$. Mean value of pre-WBC is significantly higher in *Spirulina* than in *Chlorella*, $p= 0.001$.

4.3.2.1 Post- *A- hydrophila* challenge

Similarly, there was no statistical mean differences between SP and CL in the post -*A. hydrophila* challenge values of Hgb at $p = 0.084$; Hct, $p = 0.87$; RBC, $p = 0.34$; MCV, $P = 0.50$, MCH, $p = 0.45$; and MCHC, $p = 0.93$. However, *A. hydrophila* significantly affected the mean values of RDW at $F (1, 12) = 17.5$, $p = 0.001$; WBC, = (49.5) $p < 0.001$, and Platelets, = (17.6) $p < 0.001$. Mean value of both post-RDW and WBC of *Spirulina* was higher than that of *Chlorella*, $p = 0.001$ while in that of platelets value, *Chlorella* was significantly higher than *Spirulina*, $p = 0.001$.

4.3.2.2 Pre- *A. hydrophila challenge*

There was a significant mean difference of pre-Hgb value between dietary levels, $F (2, 12) = 65.08$, $p < 0.001$. Mean values of 50% and 75% inclusion levels were significantly higher than control (0% algae) at $p < 0.001$ whereas there was no significant difference between 50% and 75%, $p = 1.00$. There was, however, no significant interaction, $p = 0.99$ between Algae type × dietary inclusion levels (AL× DL).

There was a significant mean difference of pre-Hct between the dietary level, $F (2,12) = 42.1$, $p < 0.001$. Mean values of 50% and 75% inclusion levels were significantly

higher than control (0% algae) at $p < 0.001$ whereas there was no significant difference between 50% and 75%, $p = 1.00$ and no significant interaction, $p = 0.99$ between Algae type × dietary inclusion levels.

There was a significant mean difference of pre-RBC between diet level, $F(1, 12) = 6.6$, $p = 0.011$. Equally, mean values of 50% and 75% inclusion levels were significantly higher than control (0% algae) at $p < 0.001$ while there was no significant difference and interaction between 50% and 75% and algae type × dietary inclusion levels at $p = 1.00$ and 0.99 respectively.

As shown in the main two-way ANOVA output, no significant differences and interaction were observed between dietary level, $p = 0.23$; 0.92 for MCV and $p = 0.92$ and 0.96 for MCH. As for pre-MCHC, a significant mean difference for diet level at, $F(2, 12) = 40.5$, $p < 0.001$ was observed and both 50% and 75% levels were significantly higher than 0% algae $p < 0.001$. Also, no significant difference between 50% and 75%, $p = 1.00$ and no significant interaction for algae type and diet level, $p = 0.59$. Similarly, pre- RDW achieved a statistical difference for diet level at, $F(2, 12) = 5.5$, $p = 0.02$. 75% was significantly lower than 0%algae, $p = 0.02$, whereas there was no significant difference between 50% and 0%, $p = 0.099$, and 50% and 75%, $p = 1.00$. No significant interaction was observed for algae type and level, $p = 0.92$.

Just like pre-WBC for algae type, there was a significant mean difference of pre-WBC for diet level, $F(2, 12) = 253.5$, $p < 0.001$. Both 50% and 75%were significantly higher than 0%, $p < 0.001$. There was no significant interaction for algae type and diet level, $p = 0.42$.

4.3.2.3 Post-*A. hydrophila challenge*

There was significant mean difference of post-Hgb value between the dietary level, $F(2, 12) = 261.9$, $p < 0.001$. 50% and 75% were significantly higher than 0%, $p <$

0.001, no significant difference between 50% and 75%, p = 1.00 and no significant interaction between algae type and dietary level, p = 0.44. Post-Hct mean was statistically significant between diet level, F (2, 12) = 77.4, p <0.001, 50% and 75% were significantly higher than 0%, p < 0.001, whereas 50% and 75% achieved no significant difference p = 1.00. No significant interaction, p = 0.99 between AT× DL was also observed.

Likewise, post-RBC mean values scored statistical significance between diet level, F (2, 12) = 63.3, p < 0.001. 50% and 75% were significantly higher than 0%, p < 0. 001 whereas, no significant difference between 50% and 75%, p = 1.00 and no AT× DL interaction recorded, p = 0.27. For MCV, there was a significant mean difference between diet level, F (2, 12) = 5.6, p = 0.02. 50% was significantly lower than 0%, p = 0.03, no significant mean difference between 75% and 0%, p = 0.06 and between 50% and 75%, p = 1.00. There was also no observable significant interaction for AT× DL, p = 0.47.MCH also recorded a significant mean difference for diet level, F (2, 12) = 6.9, p = 0.01,50% and 75% were significantly lower than 0%, p = 0.017 and 0.030 respectively. There was no significant difference between 50% and 75%, p = 1.00 nor AL ×DL interactions. Post MCHC recorded no significant mean differences for diet level, p = 0.95 nor interaction for AL and DL, p = 0.88 and none of the paired samples were significantly different, all p values = 1.00.

There was a significant difference of post-RDW for diet level, F (2, 12) = 1115.0, p < 0.001. 50% and 75% was significantly lower than 0%, p < 0.001and the interaction between algae type and diet level was significant, F (2, 12) = 18.1, p < 0.001. *Spirulina* 50% was significantly higher than 75%, p = 0.001 but for *Chlorella* 50% was significantly lower than 75%, p = 0.004.

Just like in effect of algae type, there was a significant mean difference of post-WBC value for diet levels, p < 0.001. Both 50% and 75% levels were significantly lower than 0%, p < 0.001. 75% is significantly higher than 50%, p = 0.03. There was a

significant interaction, $p < 0.001$ between AT ×DL. All the pairs were significantly different from each other, all p values less than 0. 001. For *Spirulina* 50% was significantly higher than 75%, $p < 0.001$, while for *Chlorella* 50% was significantly lower than 75%, $p < 0.001$.

For platelets mean values, there was also a significant mean difference for diet level, $p < 0.001$. 50% and 75% levels were significantly higher than 0%, $p < 0.001$.50% was significantly higher than 75%, $p < 0.001$. There was a significant interaction between algae type and diet level, $p < 0.001$. All paired samples were statistically different, and the difference was all in the same direction.

Table 4.11: Two-way ANOVA for algae type and dietary level on hematological parameters (pre and post)

TREATMENT	Hgb (g/dl)	HCT %	RBC (106cells/mm3)	MCV (fl)	MCH (pg.)	MCHC (g/dl)	RDW (%)	WBC (103 cells/mm3)	PLTLT (103 cells/mm3)
Algae type									
Spirulina Pre	12.10±0.03	46.18±0.07	3.99±0.0	115.88±0.81	30.36±0.18	26.20±0.04	12.47±0.02	93.15b±0.02	7.00±0.16
Spirulina Post	11.22±0.02	42.02±0.16	3.31±0.02	127.28±0.98	33.97±0.25	26.70±0.11	14.24±0.02 [b]	97.48±0.03 [b]	10.00±011 [a]
Chlorella Pre	12.07± 0.03	46.17±0.07	3.97±0.03	116.47±0.81	30.48±0.18	26.13± 0.04	12.46±0.02	93.05±0.02 [a]	6.89±0.16
Chlorella Post	11.24±0.02	42.06± 0.16	3.34±0.02	126.31±0.98	33.70±0.25	26.68±0.11	14.11±0.02 [a]	97.21±0.03 [a]	10.65±0.11 [b]
Dietary levels									
0% Algae Pre	11.74±0.04 [a]	45.51±0.09 [a]	3.87±0.04 [a]	117.62±0.99	30.35±0.22	25.81±0.05 [a]	12.55±0.03 [a]	92.72±0.02 [a]	7.83±0.19 [c]
0% Algae Post	10.64±0.03 [a]	40.02±0.19 [a]	3.08±0.03 [a]	130.04b±1.20	34.75±0.30	26.73±0.14	15.16b±0.03	99.03c±0.03	7.98±0.14 [a]
50% Algae Pre	12.25±0.04 [b]	46.50±0.09 [b]	4.02±0.04 [b]	115.68±0.99	30.46±0.22	26.34±0.05 [b]	12.44±0.03	93.31b±0.02	7.00b±0.19
50% Algae Post	11.48±0.03 [b]	43.04±0.19 [b]	3.45b±0.03	124.90±1.20 [a]	33.32±0.30	26.68±0.14	13.70±0.03 [a]	96.44±0.03 [a]	13.50±0.14 [c]
75% Algae Pre	12.27±0.04 [b]	46.53±0.09 [b]	4.04±0.04 [b]	115.23±0.99	30.45±0.22	26.36±0.05 [b]	12.41±0.03 [a]	93.28±0.02 [b]	6.00±0.19 [a]
75% Algae Post	11.48±0.03 [b]	43.03±0.19 [b]	3.44±0.03 [b]	125.45±1.20 [a]	33.45±0.30	26.67±0.14	13.67±0.03 [a]	96.58±0.03 [b]	9.50±0.14 [b]

Table 4.11, continued

TREATMENT	Hgb (g/dl)	HCT %	RBC (106cells/mm3)	MCV (fl)	MCH (pg.)	MCHC (g/dl)	RDW (%)	WBC (103 cells/mm3)	PLTLT (103 cells/mm3)
Algae × level									
SP × 0% Pre	11.78±0.05	45.50±0.13	3.89±0.05	117.00±1.49	30.28±0.31	25.88±0.07	12.56±0.05	92.75±0.03	8.00±0.28
SP ×0% post	10.70±0.04	40.00±0.28	3.08±0.04	129.78±1.70	34.72±0.43	26.76±0.19	15.18c±0.04	99.00±0.05 [c]	8.00±0.19 [a]
SP × 50% Pre	12.25±0.05	46.50±0.13	4.02±0.05	115.00±1.49	30.45±0.31	26.34±0.07	12.45±0.05	93.36±0.03	7.00±0.28
SP × 50% post	11.45±0.04	43.00±0.28	3.40±0.04	126.62±1.70	33.72±0.43	26.63±0.19	13.88b±0.04	96.87±0.05 [b]	12.00c±0.19
SP ×75% Pre	12.28±0.05	46.55± 0.13	4.05±0.05	115.04±1.49	30.35±0.31	26.38±0.07	13.40±0.05	93.34±0.03	6.00±0.28
SP × 75% Post	11.50±0.04	43.07±0.28	3.44±0.04	125.43±1.70	33.49±0.43	26.70±0.19	13.65±0.04 [a]	96.56±0.05 [a]	10.00±0.19 [b]
CL × 0% Pre	11.71±0.05	45.51±0.13	3.85±0.05	118.25±1.49	30.42±0.31	25.73±0.07	12.54±0.05	92.70±0.03	7.67±0.28
CL × 0% Post	10.69±0.04	40.04±0.28	3.07±0.04	130.30±1.70	34.78±0.43	26.69±0.19	15.14c±0.04	99.05±0.05 [b]	7.96±0.19 [a]
CL × 50% Pre	12.24±0.05	46.49±0.13	4.02±0.05	115.75±1.49	30.48±0.31	26.33±0.07	12.43±0.05	93.25±0.03	7.00±0.28
CL ×50% Post	11.52±0.04	43.09±0.28	3.50±0.04	123.18±1.70	32.92±0.43	26.73±0.19	13.51±0.04 [a]	96.00±0.05 [a]	15.00c±0.19
CL × 75% Pre	12.26±0.05	46.51±0.13	4.03±0.05	115.42±1.49	30.55±0.31	26.33±0.07	12.41±0.05	93.21±0.03	6.00±0.28
CL ×75% Post	11.52±0.04	43.06±0.28	3.43±0.04	125.46±1.70	33.42±0.43	26.30±0.19	13.69b±0.04	96.59±0.05 [c]	9.00b±0.19

Mean values (n = 18) in a column under each category of parameter bearing different superscript (lower case) vary significantly (P < 0.05).

4.3.3 Effects of algae types, inclusion levels and their interactions on biochemical parameters pre and post *A. hydrophila challenge*

Assumption for equality of variance using Levene's test: All the assumptions were met ($p > 0.05$), except for Post Globulin = 0.034 and Post A/G ratio = 0.047.

4.3.3.1 Pre- *A. hydrophila challenge*

The main effect of two-way ANOVA shows that there was a significance mean difference of pre-glucose, AL, Trig, TCL and HDL between the two types of algae, F (1, 12) = 708.48, $p < 0.001$; 159.86,

$p < 0.001$; 1835.62, $p < 0.001$; 18.29, $p = 0.001$ and 53.25, $p < 0.001$ respectively. There were however no significant differences $p > 0.05$ in TSP, Alb, Glob, A/G ratio, ALP, AST, Crt and LDL cholesterol.

4.3.3.2 Post- *A. hydrophila challenge*

Remarkable differences were observed post-*A. hydrophila* in the mean values of glucose; ALP, AL, Trig, TCL, HDL and LDL between the two types of algae at, F (1, 12) = 210.79, $p < 0.001$; 14.53, $p = 0.002$; 201.32, $p < 0.001$; 803.83, $p < 0.001$; = 11.66, $p = 0.005$; 86.70, $p < 0.001$ and 100.89, $p < 0.001$ correspondingly. However, there were no significant $p > 0.05$ differences in TP, Alb, Glob, A/G ratio, AST and Crt.

4.3.4 Effects of dietary inclusion levels on biochemical parameters pre and post *A. hydrophila challenge*

4.3.4.1 Pre- *A. hydrophila challenge*

There is a significant mean difference of pre-glucose, TSP, globulin, A/G ratio, AST, AL, Crt , Trig, TCL, HDL and LDL cholesterol between the dietary levels, F (2, 12) = 4984.88, $p < 0.001$; = 61.52, $p < 0.001$; = 98.58, $p < 0.001$; = 11.09, $p = 0.002$; = 2114.43, $p < 0.001$; = 2919.22, $p < 0.001$; = 8.26, $p = 0.006$; = 44193.12, $p < 0.001$; =

34738.41, $p < 0.001$; = 37089.30, $p < 0.001$ and = 173.15, $p < 0.001$ respectively. There were however, no remarkable differences in the mean values obtained for Alb and ALP.

4.3.4.2 Post- *A. hydrophila challenge*

Post- *A. hydrophila* challenge, caused significant mean differences between the dietary levels, $F(2, 12) = 31485.63$, $p < 0.001$;) = 1536.0, $p < 0.001$;) = 568.02, $p < 0.001$;) = 84.20, $p < 0.001$; = 616.86, $p < 0.001$; = 114.96, $p < 0.001$; = 5642.17, $p < 0.001$;) = 7.14, $p = 0.009$; = 202.54, $p < 0.001$; = 10561.11, $p < 0.001$; = 117830.00, $p < 0.001$ and = 30043.35, $p < 0.001$;for glucose ,Alb, Glob, A/G ratio, ALP, AST, AL, Crt, Trig, TCL, HDL and LDL cholesterol respectively.

4.3.5 Interaction (Algae type × dietary level)

4.3.5.1 Pre- *A. hydrophila challenge*

There was a significant interaction term between algae type and dietary level for glucose, AL, TRIG, TCL, HDL and LDL, whereas there was for TSP, Alb, glob, A/G ratio, AST, Crt, ($p > 0.005$).

4.3.5.2 Post- *A. hydrophila* challenge

Post-*A. hydrophila* challenge produced a significant interaction term between algae type and dietary level for glucose, TSP, Alb, glob, AL, Trig, HDL and LDL, ($p < 0.005$) and non-occurred for A/G ratio, ALP, AST, Crt, and TCL, ($p > 0.005$)

Table 4.12: Effects of algae, inclusion levels and their interactions on biochemical parameters of *C. gariepinus* Pre and post *A. hydrophila* challenge

Biochemical parameters	*Spirulina*	*Chlorella*	Control	50%	75%	SP× control	SP× 50%	SP × 75%	CL× control	CL×50%	CL × 75%
PG Pre (mg/dl)	74.32±0.01^{B}	73.94±0.01^{A}	77.34±0.01^{C}	72.66±0.01^{B}	72.38±0.01^{A}	77.31±0.02^{C}	72.87±0.02^{B}	72.78±0.02^{A}	77.37±0.02^{C}	72.45±0.02^{B}	71.98±0.02^{A}
PG Post (mg/dl)	75.29±0.01^{B}	74.98±0.01^{A}	78.89±0.02^{C}	73.41±0.02^{B}	73.10±0.02^{A}	78.86±0.03^{C}	73.78±0.03^{B}	73.22±0.03^{A}	78.92±0.03^{C}	73.04±0.03^{B}	72.97±0.03^{A}
TSP Pre (g/dl)	4.47±0.03	4.45±0.03	4.18±0.03^{A}	4.58±0.03^{B}	4.63±0.03^{B}	4.20±0.04	4.58±0.04	4.64±0.04	4.17±0.04	4.57±0.04	4.61±0.04
TSP Post (g/dl)	3.67±0.02	3.71±0.02	2.98±0.02^{A}	4.04±0.02^{B}	4.06±0.02^{B}	3.00±0.03^{A}	3.95±0.03^{B}	4.06±0.03^{C}	2.96±0.03^{A}	4.13±0.03^{C}	4.05±0.03^{B}
ALB Pre (g/dl)	1.79±0.02	1.79±0.02	1.76±0.03	1.80±0.03	1.81±0.03	1.75±0.04	1.80±0.04	1.82±0.04	1.77±0.04	1.79±0.04	1.80±0.04
ALB Post (g/dl)	1.50±0.01	1.51±0.01	1.30±0.01^{A}	1.62±0.01^{B}	1.62±0.01^{B}	1.30±0.01^{A}	1.59±0.01^{B}	1.62C±0.01	1.29±0.01^{A}	1.64±0.01^{C}	1.61±0.01^{B}
Glob Pre (g/dl)	2.68±0.02	2.66±0.02	2.43±0.02^{A}	2.78±0.02^{B}	2.82±0.02^{B}	2.45±0.03	2.78±0.03	2.82±0.03	2.40±0.03	2.78±0.03	2.81±0.03
Glob Post (g/dl)	2.17±0.01	2.20±0.01	1.69±0.02^{A}	2.43±0.02^{B}	2.44±0.02^{B}	1.70A±0.03	2.36±0.03^{B}	2.44±0.03^{C}	1.67A±0.03	2.49±0.03^{B}	2.44±0.03^{B}
A/GR Pre (%)	0.67±0.01	0.67±0.01	0.73±0.01^{B}	0.65±0.01^{A}	0.64±0.01^{A}	0.71±0.02	0.65±0.02	0.65±0.02	0.74±0.02	0.64±0.02	0.64±0.02
A/GR Post (%)	0.70±0.01	0.70±0.01	0.77±0.01^{B}	0.67±0.01^{A}	0.66±0.01^{A}	0.76±0.01	0.67±0.01	0.66±0.01	0.77±0.01	0.66±0.01	0.66±0.01
ALP Pre (IU/L)	9.87±0.03	9.84±0.03	9.84±0.04	9.85±0.04	9.87±0.04	9.83±0.05	9.88±0.05	9.89±0.05	9.86±0.05	9.81±0.05	9.84±0.05
ALP post (IU/L)	10.33±0.02^{A}	10.43±0.02^{B}	9.70±0.02^{A}	10.68±0.02^{B}	10.76±0.02^{C}	9.63±0.03	10.65±0.03	10.70±0.03	9.77±0.03	10.71±0.03	10.82±0.03
AST Pre (IU/L)	60.98±0.02	60.98±0.02	62.23±0.02^{B}	60.35±0.02^{A}	60.36±0.02^{A}	62.20±0.03	60.36±0.03	60.37±0.03	62.25±0.03	60.33±0.03	60.35±0.03
AST post (IU/L)	62.56±0.15	62.18±0.15	64.58±0.18^{B}	61.45±0.18^{A}	61.10±0.18^{A}	64.50±0.25	62.00±0.25	61.19±0.25	64.65±0.25	60.89±0.25	61.00±0.25

Table 4.12, continued

Biochemical parameters	*Spirulina*	*Chlorella*	Control	50%	75%	SP× control	SP× 50%	SP × 75%	CL× control	CL×50%	CL × 75%
AL Pre (IU/L)	23.09±0.03^{B}	22.62±0.03^{A}	24.84±0.03^{C}	22.10±0.03^{B}	21.62±0.03^{A}	24.82±0.05^{C}	23.20±0.05^{B}	21.24±0.05^{A}	24.85±0.05^{C}	21.00±0.05^{A}	22.00±0.05^{B}
AL Post (IU/L)	23.55±0.02^{B}	23.17±0.02^{A}	25.35±0.02^{C}	22.66±0.02^{B}	22.08±0.02^{A}	25.30±0.03^{C}	23.80±0.03^{B}	21.56±0.03^{A}	25.39±0.03^{C}	21.53±0.03^{A}	22.60±0.03^{B}
Crt Pre (mg/dl)	0.35±0.01	0.35±0.01	0.33±0.01^{A}	0.36±0.01^{B}	0.36±0.01^{B}	0.33±0.01	0.35±0.01	0.36±0.01	0.33±0.01	0.36±0.01	0.35±0.01
Crt Post (mg/dl)	0.31±0.01	0.31±0.01	0.28±0.01^{A}	0.33±0.01^{B}	0.33±0.01^{B}	0.29±0.01	0.32±0.01	0.33±0.01	0.27±0.01	0.33±0.01	0.33±0.01
Trig Pre (mg/dl)	83.88±0.03^{B}	82.34±0.03^{A}	90.66±0.03^{C}	79.53±0.03^{B}	79.16±0.03^{A}	90.61±0.04^{C}	80.69±0.04^{B}	80.35±0.04^{A}	90.71±0.04^{C}	78.36±0.04^{A}	77.96±0.04^{B}
Trig Post(mg/dl)	78.73±0.03^{B}	77.63±0.03^{A}	78.73±0.03^{B}	77.89±0.03^{A}	77.93±0.03^{A}	78.76±0.05	78.69±0.05	78.73±0.05	78.70±0.05^{B}	77.08±0.05^{A}	77.12±0.05^{A}
TCL Pre (mg/dl)	95.27±0.02^{B}	95.16±0.02^{A}	99.87±0.02^{C}	92.97±0.02^{B}	92.82±0.02^{A}	99.81±0.03^{C}	93.05±0.03^{B}	92.95±0.03^{A}	99.92±0.03^{C}	92.89±0.03^{B}	92.68±0.03^{A}
TCL Post (mg/dl)	93.63±0.02^{B}	93.53±0.02^{A}	96.52±0.02^{B}	92.09±0.02^{A}	92.14±0.02^{A}	96.58±0.04^{C}	92.12±0.04^{A}	92.19±0.04^{B}	96.47±0.04^{C}	92.05±0.04^{A}	92.08±0.04^{B}
HDL Pre (mg/dl)	65.82±0.02^{A}	66.05±0.02^{B}	59.81±0.03^{A}	68.06±0.03^{B}	69.93±0.03^{C}	59.86±0.04^{A}	66.93±0.04^{B}	70.66±0.04^{C}	59.77±0.04^{A}	69.18±0.04^{B}	69.21±0.04^{B}
HDL Post (mg/dl)	62.79±0.02^{A}	63.02±0.02^{B}	54.39±0.02^{A}	67.28±0.02^{C}	67.05±0.02^{B}	54.44±0.03^{A}	66.87±0.03^{B}	67.06±0.03^{C}	54.33±0.03^{A}	67.69±0.03^{C}	67.04±0.03^{B}
LDL Pre (mg/dl)	58.83±0.02	58.88±0.02	59.23±0.02^{B}	58.68±0.02^{A}	58.67±0.02^{A}	59.11±0.03^{C}	58.70±0.03 ^{B}	58.68±0.03^{A}	59.34±0.03^{C}	58.66±0.03^{B}	58.65±0.03^{A}
LDL Post (mg/dl)	65.19±0.02^{B}	64.86±0.02^{A}	70.79±0.03^{C}	62.22±0.03^{B}	62.06±0.03^{A}	70.76±0.04^{B}	62.46±0.04^{A}	62.35±0.04^{A}	70.83±0.04^{C}	61.97±0.04^{B}	61.77±0.04^{A}

Mean values (18) in a row for the same parameter under algae type, dietary level and interaction for algae type and dietary level bearing different superscript (capital) vary significantly ($P < 0.05$).

4.3.6 Lysozyme Activity day 10 and 18 Post *A. hydrophila* challenge

4.3.6.1 Algae type

There was a significant mean difference of lysozyme activity (LA) at 10 and 18 days post challenge between the two algae, $F(1, 12) = 20.53$, $p = 0.01$ and $= 422.51$, $p < 0.001$ respectively. Paired sample T-test revealed a statistical mean difference of between days10 days and 18 lysozyme activities for *Spirulina*, $p < 0.001$and for *Chlorella*, $p < 0.001$. Day 18 LA was however significantly higher than at 10 days for all the algae.

4.3.6.2 Dietary inclusion levels effect on days 10 and 18

There was also a significant mean difference of both day 10 and 18 LA between the dietary inclusion levels, $p < 0.001$. Also paired sample T-test showed a significant mean difference between days 10 and 18 LA for control (0 algae), $p < 0.001$; for 50%, $p < 0.001$; for 75% inclusion levels, $p < 0.001$. Like the algae type, day 18 LA was significantly higher than that of day 10 for all the dietary inclusion levels.

4.3.6.3 The interaction between algae type × dietary inclusion levels

There was a significant interaction between algae type and dietary inclusion level for days 10 and 18 LA at $p = 0.002$ and $p < 0.001$ correspondingly as shown in Table 4.13.

Table 4.13: Two-way ANOVA for the effect of algae type and dietary inclusion levels on days 10 and 18 post- *A. hydrophila* challenge on catfish lysozyme activity

Treatment	Day10 Lysozyme activity	Day18 Lysozyme activity
Algae type		
Spirulina	120.33 ± 0.24^{Aa}	139.05 ± 0.03^{Ab}
Chlorella	121.86 ± 0.24^{Ba}	139.96 ± 0.03^{Bb}
Inclusion levels		
Control	112.96 ± 0.29^{Aa}	132.68 ± 0.4^{Ab}
50% meal	123.50 ± 0.29^{Ba}	142.10 ± 0.04^{Bb}
75% meal	126.84 ± 0.29^{Ca}	143.74 ± 0.04^{Cb}
Algae type x inclusion levels		
Spirulina x control	113.00 ± 0.41^{Aa}	132.67 ± 0.05^{Ab}
Spirulina x 50% meal	123.00 ± 0.41^{Ba}	142.00 ± 0.05^{Bb}
Spirulina x 75%	125.00 ± 0.41^{Ca}	142.47 ± 0.05^{Cb}
Chlorella x control	112.92 ± 0.41^{Aa}	132.68 ± 0.05^{Ab}
Chlorella x 50%	124.00 ± 0.41^{Ba}	142.20 ± 0.05^{Bb}
Chlorella x 75%	128.67 ± 0.41^{Ca}	145.00 ± 0.05^{Cb}

Mean values (n = 18) in same column (capital) under each parameter bearing different superscript differ significantly ($p < 0.005$).
Mean values (n = 18) in the same row (10 and 18 days) for each stimulant bearing different superscript (small) differ significantly.

4.3.7 Respiratory burst activity (RBA) day 10 and 18 post *A. hydrophila challenge*

4.3.7.1 Algae type

There were significant mean differences of day 10 and 18 RBA activity between the two algae, $F(1, 12) = 48.19$, $p < 0.001$ and $= 16.35$, $p = 0.002$.

4.3.7.2 Dietary inclusion level

There were significant mean differences of RBA activity at days 10 and 18th between the dietary inclusion level, $F(2, 12) = 726.00$, $p < 0.01$ and $= 258.19$, $p < 0.01$.

4.3.7.3 Dietary interaction for algae and diet inclusion level

There was a significant interaction between algae and dietary inclusion level for both RBA activities at 10 and 18 days, $p < 0.001$. The reasons for interaction for both day 10 and 18 from the Table 4.14 being the fact that the control was significantly $p < 0.05$ lower than 50% meal and 75% inclusion levels of *Spirulina* meals, but no significant $p >$

0.05 difference between 50% meal and 75% meal. For *Chlorella* meals, however, control was significantly lower than 50% meal and 75% meal, and 75% meal inclusion level was significantly lower than 50%.

4.3.7.4 Comparison of days 10 and 18 RBA activities

A paired t-test comparing days 10 and 18 was conducted. As shown in Table 4.14, there was no significant mean difference between RBA at 10 days and at 18 days for both *Spirulina* and *Chlorella*, and all the dietary inclusion levels $P > 0.005$. For interaction term between algae type and dietary inclusion levels, however, and as indicated in (Table 4.14) by different superscript in small letters, only the mean of RBA for *Chlorella* × 75% meal showed a significant difference ($p < 0.013$) between 10 days and 18 days post *A. hydrophila* challenge.

Table 4.14: Two-way ANOVA for the effect of RBA at 10 and 18 days on algae type and dietary inclusion levels

Treatment	RBA activity at 10 days	RBA activity at 18 days
Algae type		
Spirulina	0.81 ± 0.01^{A}	0.84 ± 0.01^{A}
Chlorella	0.94 ± 0.01^{B}	0.94 ± 0.01^{B}
Dietary inclusion levels		
Control	0.43 ± 0.02^{A}	0.46 ± 0.03^{A}
50% meal	1.28 ± 0.02^{C}	1.33 ± 0.03^{C}
75% meal	0.91 ± 0.02^{B}	0.94 ± 0.03^{B}
Algae type x inclusion levels		
Spirulina x control	0.42 ± 0.02^{A}	0.43 ± 0.04^{A}
Spirulina x 50% meal	1.02 ± 0.02^{B}	1.10 ± 0.04^{B}
Spirulina x 75%	0.99 ± 0.02^{B}	1.00 ± 0.04^{B}
Chlorella x control	0.45 ± 0.02^{A}	0.48 ± 0.04^{A}
Chlorella x 50%	1.53 ± 0.02^{C}	1.55 ± 0.04^{C}
Chlorella x 75%	0.83 ± 0.02^{Ba}	0.88 ± 0.04^{Bb}

Mean values (n = 18) in same column (capital) under each parameter bearing different superscript differ significantly ($p < 0.005$). RBA: Respiratory burst activity

4.3.8 Percentage cumulative mortality of experimental fish 18 days post- *A. hydrophila challenge*

The assumption for homogeneity of variance was met $p = 0.77$. There was a significant difference in the average mortality rate between the diet treatment level, $F(4, 10) = 107.67$, $p < 0.001$. All the dietary levels were significantly different, $p < 0.05$ from one another with 50% *Chlorella* having the lowest average mortality rate (26.67%) and control having the highest average mortality rate (80%).

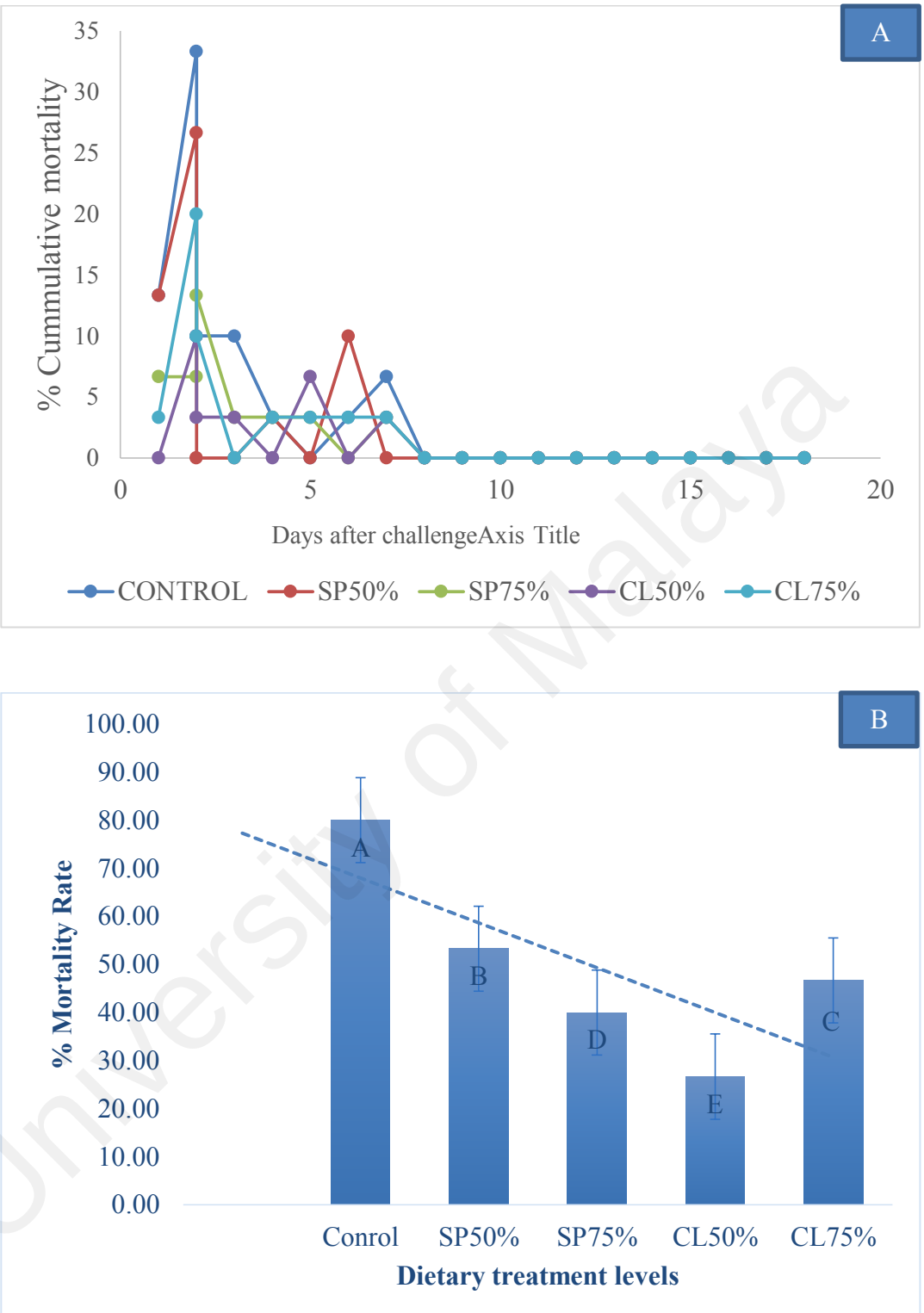


Figure 4.3: (A) % Daily cumulative mortality in *C. gariepinus* Fed *Spirulina* and *Chlorella* after *A. hydrophila challenge* and (B) % mean cumulative mortality in *C. gariepinus* Fed *Spirulina* and *Chlorella* after *A. hydrophila challenge*

4.4 The effects of dietary *Spirulina* and *Chlorella* on growth, fillet composition and antioxidant activities of Africa catfish (*C. gariepinus*)

4.4.1 Fish gross composition of experimental diets

Table 4.15 presents the gross composition of the experimental diet and nutrient, which reveals that all the diets are iso-nitrogenous (35%) and iso-energetic (417 Kcal 100g-1). The crude protein, crude lipid, and moisture content decreased, while the ash and fibre levels increased with increase in the level of supplementation of both *S. platensis* and *C. vulgaris*.

4.4.2 Amino acid profile of experimental diets

Table 3.9 presents the amino acid profile of the experimental diets containing graded levels of *S. platensis* and *C. vulgaris*. There is no significant difference in the tryptophan value of all the diets including the control diet. Supplementation with *C. vulgaris* has no significant effect on the value of arginine in the diet but it increased upon supplementation with *S. platensis*. Supplementation with *S. platensis* and *C. vulgaris* reduced the values of glycine, cysteine, and methionine in the diets. The value of aspartic acid, Histidine and tyrosine decreased upon addition of *C. vulgaris* to the diet, while their values increased upon addition of *S. platensis*. Furthermore, the value of all other amino acids significantly increased upon the inclusion of *S. platensis* and *C. vulgaris* in the diet.

4.4.3 Fatty acid profiles of experimental diets

Table 3.10 presents the FA profile of the iso-nitrogenous and isoenergetic experimental diets containing graded levels of *S. platensis* and *C. vulgaris*. High level of *S. platensis* favoured the high concentration of FA except for the sum of n-9 and n-3 FA. The fatty acid content was highest at 75% *S. platensis* supplementation. This indicates that a high level of supplementation of *S. platensis* resulted in a higher total content of

monounsaturated, PUFA, as well as saturated fatty acid. Similarly, a high level of *C. vulgaris* engendered high concentration of FA except for saturated and the sum of n-3 FA. The FA content was highest at 75% *C. vulgaris* supplementation. This indicates that a high level of supplementation of *C. vulgaris* resulted in a higher total content of monounsaturated and PUFA. Comparing the two supplements, SP75 has the highest content of PUFA, SFA, ∑n-6, and C18:3 n-4, while CL75 has the highest amount of ∑n-3, ∑n-9, DPA (docosahexaenoic acid), as well as the n-3/n-6 ratio. They both have a similar amount of MUFA and EPA.

4.4.4 Fish liver protein and enzymatic CAT, GST and SOD activities

Table 4.15 presents the liver protein, GST, CAT, and SOD activity of African catfish Fed with *S. platensis* and *C. vulgaris* enriched diets GST and liver protein in all the supplemented diet displayed slightly lower activities when compared with the control diets but with insignificant differences. The enriched diets showed significantly higher CAT and SOD activities when compared with the control diet with CL75 and SP75 showing the highest activity of CAT and SOD respectively. There was, however, no significant difference between the CAT activities of both CL75 and SP75 and SOD activities of all the algae treated diet groups. No significant differences were detected in GST activity of control fish group with the level of diet supplementation for both *S. platensis* and *C. vulgaris,* but control showed the highest activity. The activities of liver protein decreased upon incorporation of *S. platensis* and *C. vulgaris* but further increase in the level of incorporation yield no significant change.

4.4.5 Correlation between liver catalase activities and mean weight gain of fishes

Figure 4.4 A & B illustrates the correlation between liver catalase activities, and mean weight gain of African catfish Fed with *S. platensis* and *C. vulgaris* supplemented

diets. The liver catalase activities of both *S. platensis* and *C. vulgaris* are characterized by increasing weight gain.

Table 4.15: Liver protein (mg ml^{-1}), Catalase (CAT), Superoxide dismutase (SOD) and Glutathione s-transferase (nmol mg^{-1}protein) activity of African catfish Fed *Spirulina* and *Chlorella* diets.

Enzymes and Liver Protein	Control	SP50%	SP75%	CL50%	CL75%	P Value
Liver protein	0.67 ± 0.01 [a]	0.60 ± 0.03 [b]	0.61 ± 0.01 [b]	0.61 ± 0.01 [b]	0.62 ± 0.01 [b]	.044
CAT	200.71 ±0.01[c]	244.41 ± 0.52 [b]	256.00 ± 0.58 [a]	243.51 ±0.40 [b]	256.81 ± 0.50 [a]	.0001
GST	175.87±0.03 [a]	175.80 ± 0.06 [a]	175.82 ± 0.05 [a]	175.81 ± 0.06 [a]	175.83 ± 0.05[a]	.874
SOD	33.54 ± 0.06[b]	42.33± 0.11 [a]	42.35 ± 0.06 [a]	42.32 ± 0.06 [a]	42.34 ± 0.06 [a]	.0001

Mean ± SE of fifteen fish per dietary treatment, five fish/ tank. Mean values in the same row with different superscript (a -c) are statistical different ($p < 0.05$))

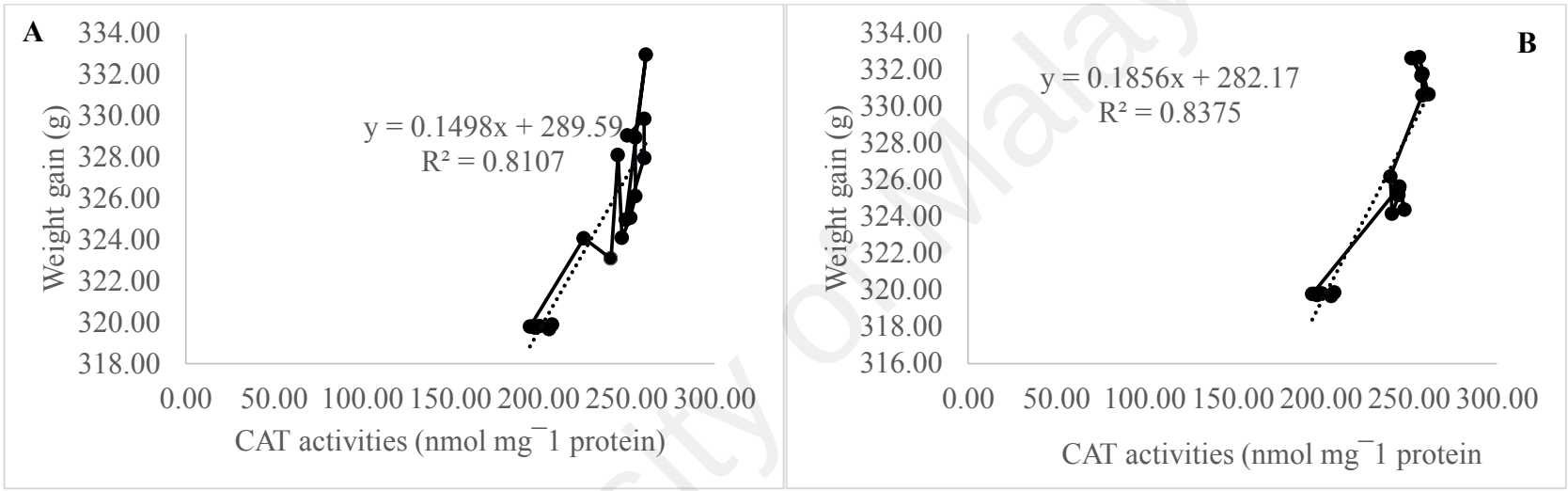


Figure 4.4: A & B Correlation between catalase activity and mean weight gain of fish Fed *Spirulina* and *Chlorella* supplemented diets. The results indicate the mean ± standard error (SE) of five fish/tank (15 fish per treatment)

4.4.6 Growth performance of *C. gariepinus* fed experimental diets

Table 4.16 presents the assessment growth performance of juvenile *C. gariepinus* Fed with graded levels of *S. platensis* and *C. vulgaris*. The outcomes showed that *S. platensis* and *C. vulgaris* are good replacements for fishmeal as a source of protein in the diet of African catfish. The feed intake reduced, while the SGR increased upon supplementation with *S. platensis* and *C. vulgaris,* but it was not significant. The body weight gain (BDWG) increased significantly as the level of supplementation increased. The highest BDWG was observed with CL75, which also exhibit the best FCR and PER. The best protein productive value (PPV) was obtained at 50% replacement for both *S. platensis* and *C. vulgaris*. A further increase to 75% led to a significant decrease in PPV for *S. platensis* but insignificant for *C. vulgaris*. The value of the K factor and HIS increased significantly upon supplementation with *S. platensis* and *C. vulgaris*, and the highest values were observed with CL75. There was no mortality recorded throughout the experimental period.

4.4.7 Fillets composition of *C. gariepinus* diets

Table 4.17 presents the fillet composition of the experimental fishes. The crude protein, crude lipid, and ash moisture content decreased in a dose-dependent manner, while the moisture and fibre levels increased with increase in the level of supplementation of both *S. platensis* and *C. vulgaris*.

Table 4.16: Growth performance of *C. gariepinus* juveniles Fed with graded levels of *Spirulina* and *Chlorella*

Variables	Control	SP50	SP75	CL50	CL75	P
FW	361.89±0.03^{c}	367.16±0.03^{b}	371.01±0.05^{ab}	367.26±0.43^{b}	374.08±0.29^{a}	.001
IW	42.11±0.18^{a}	42.07±0.03^{a}	42.05±0.16^{a}	42.08±0.04^{a}	42.03±0.03^{a}	.988
BDWG	319.78±0.02^{c}	325.09±1.54^{b}	328.96±2.31^{ab}	325.18±0.41^{b}	332.05±0.59^{a}	.001
FI	249.66±0.66^{a}	249.14±0.06^{a}	249.42±0.07^{a}	249.21±0.03^{a}	249.01±0.01^{a}	.582
FCR	0.80±0.01^{a}	0.77±0.01^{b}	0.76±0.01^{b}	0.77±0.01^{b}	0.75±0.01^{b}	.022
SGR	5.86±0.01^{a}	5.87±0.01^{a}	5.88±0.01^{a}	5.87±0.01^{a}	5.89±0.01^{a}	.267
PER	3.03±0.01^{c}	3.11±0.02^{b}	3.13±0.02^{b}	3.11±0.01^{b}	3.18±0.01^{a}	.000
PI	105.51±0.36^{a}	104.64±0.04^{bc}	105.18±0.07^{ab}	104.64±0.04^{bc}	104.51±0.04^{c}	.007
PPV	10.81±0.05^{e}	13.11±0.01^{a}	12.59±0.01^{b}	12.37±0.01^{c}	12.35±0.01^{c}	.000
PR	11.41±0.02^{e}	13.72±0.02^{a}	13.24±0.02^{b}	12.94±0.02^{c}	12.80±0.02^{d}	.000
K Factor	1.46±0.07^{b}	1.55±0.04^{ab}	1.56±0.06^{ab}	1.55±0.01^{ab}	1.62±0.01^{a}	.268
HSI	1.43±0.01^{c}	1.59±0.03^{b}	1.58±0.02^{b}	1.59±0.02^{b}	1.70±0.01^{a}	.000
% Survival	100	100	100	100	100	

Values displayed represents the means ± SE of 5 fish/ tank with a total of fifteen fish per diet. Mean values within the same row with different superscripts are significantly different. FCR: Feed efficiency ratio; SGR: Specific growth rate; PER: Protein efficiency ratio; PI: Protein intake; PPV: Protein productive value; PR: Protein retention; K Factor: Fulton K condition factor; HIS: Hepatosomatic index.

Table 4.17: Fillet composition of Initial and *C. gariepinus* juveniles Fed with *Spirulina* and *Chlorella* diets (%)

Nutrient	Initial	Control	SP50%	SP75%	CL50%	CL75%	P
Protein	73.35±0.01 ^{d}	84.76±0.08 ^{c}	87.07±0.56 ^{a}	86.59±0.01 ^{ab}	86.29±0.07 ^{b}	86.15±0.07^{b}	.001
Lipid	8.52±0.01 ^{a}	6.01±0.01 ^{b}	4.78±0.01 ^{d}	4.00±0.01 ^{e}	5.44±0.01 ^{c}	4.80±0.01 ^{d}	.001
Fibre	0.19±0.01 ^{a}	0.17±0.02 ^{a}	0.05±0.01 ^{c}	0.09±0.01 ^{b}	0.03±0.01 ^{d}	0.07±0.01^{b}	.001
Ash	9.98±0.01 ^{a}	4.75±0.01 ^{b}	4.60±0.12 ^{c}	4.44±0.01 ^{cd}	4.48±0.02 ^{cd}	4.13±0.01^{e}	.001
Moisture	7.96±0.01 ^{a}	4.31±0.01 ^{c}	3.50±0.01 ^{e}	4.88±0.01 ^{b}	3.76±0.02 ^{d}	4.85±0.01 ^{b}	.001

Values are means of three replicates per diet ± SE. Mean with different superscript (a - e) in the same row are significant ($p < 0.05$).

CHAPTER 5: DISCUSSION OF RESEARCH FINDINGS

5.1 Results Nutrients, amino and fatty acids digestibility of dietary *Spirulina*, *Chlorella* and fishmeal in African catfish (*C. gariepinus*)

Nutrients intake by fish seldom reflects the amount that is accessible from the digestive tract, which ultimately determines the amount that is bioavailable for growth, maintenance, tissue repair and reproduction. This reality is exactly the impetus for the importance of reliable nutrient digestibility information when introducing a new feed ingredient in aquaculture. The digestibility of nutrients in two freshwater algal species, *Spirulina* and *Chlorella* were evaluated in this study to assess whether it is feasible to use them in aqua-feed. There was variation in energy content, proximate composition, fatty acid and amino acid profiles of the two microalgae. Growth performance results of this study as presented in Table 4.1 shows that the three diets containing 39.75% 41 %and 39.38% for reference, *Spirulina* and *Chlorella* respectively promoted the growth of *C. gariepinus* juveniles used in this study. *Spirulina* fed group had significantly better feed conversion ratio, weight gain and SGR than control. This finding corroborates earlier studies which reported that growth response of fish to *Spirulina* might be dependent on inclusion level as well as the nutritional composition of the diet in which it is incorporated (Abdel-Tawwab & Ahmad, 2009). *Chlorella* had the best PER which might be connected to high ADC of protein.

There are several factors that affect *in vivo* digestibility of feed ingredients by fish such as species, culture conditions, the composition of the test ingredients, diet formulation, faecal sampling and calculations (Carvalho *et al.*, 2016). Outcomes for contents and ADCs of nutrients (crude protein, lipid), AA and FA profiles suggest that *Spirulina* and *Chlorella* spp. are a potential fishmeal substitute. To the understanding of the researcher, the study seems to be the first study that examines the amino and fatty acids in addition to the

nutrient digestibility of both microalga species at the same time for *C. gariepinus.* Also, the ADC of amino acids and protein estimated in this study for fishmeal (FM), *Spirulina* (SP) and *Chlorella* (CL) feed as applied to the experimental fish were in ranges comparable to those of other ingredients tested in catfish.

Recently, Sarker *et al.* (2016) and Gong *et al.* (2017) disclosed that nutrient digestibility is different between algae sources (within a single fish species). For instance, the difference in protein digestibility may amongst others be due to changes in the inherent properties of the proteins, differences in fish species and variations in protein accessibility. The apparent digestibility of protein exceeding 90 % in all the three experimental diets (Table 4.5) in this study indicated that *C. gariepinus* juveniles could digest their proteins well. The observed ADC of crude protein in the SP and CL test ingredients (99.16% and 99.17) (Table 4.2) was higher than the protein digestibility of 80.7 and 81.4% for *Chlorella* and *Spirulina* in catfish (Teuling *et al.*, 2017) and 85.4% for cell- ruptured *Chlorella* in juveniles of Atlantic salmon (*Salmo salar L.)* (Tibbetts *et al.,* 2017*).* This findings also compares favourably with the protein ADCs of *Arthrospira* sp., in order fish species, reported to be 76%, 82% 85% and 86% in Caspian great sturgeon, Arctic charr, Atlantic salmon and Nile tilapia respectively (Burr *et al.*, 2011; Safari *et al.*, 2016; Sarker *et al.*, 2016) and 80% in *Chlorella* sp. (Sarker *et al.*, 2016). Gong *et al.* (2017) reported $ADC_{protein}$ of 67% and 72% for *Desmodessmus spp* and *Nannochloropsis sp*. in Atlantic salmon. In the last two studies above, intact algae were used, meaning that the protein and other nutrients in the cells are not directly in contact with the digestive enzymes. It is postulated that the cell walls of algae hinder nutrient accessibility, leading to a decreased nutrient digestibility. The *Chlorella* (*C. vulgaris)* used in this study had its cellulosic cell ruptured and powdered, while *Spirulina* used as with other cyanobacteria, contains peptidoglycan cell wall which is softer than the cellulose-based cell wall like *Chlorella* thus is easily digestible (Promya & Chitmanat, 2011). Furthermore, it has been recommended

that intestinal flora is improved by *S. platensis* in fish, thereby allowing break down of ingestible feed components for extraction of more nutrients and stimulates the production of enzymes that transport fats for metabolisms rather than storing within the fish (James *et al.*, 2006). A study by Khani *et al.* (2017) suggested that *Chlorella* diets increased the digestive enzymes in the pancreas and intestine of Koi carp, thereby enhancing the diet utilisation rate through an increase in the activity of digestive enzymes. Similarly, the inclusion of *Chlorella* up to 50% level in the diet of *Macrobrachium rosenbergii*, has been suggested to increase growth. Therefore, SP and CL in the current study are considered an excellent alternative protein source in *C. gariepinus* diet, compared to the earlier mentioned terrestrial plants.

Crude protein digestibility for both microalgae ingredients > 90% (Table 4.2) is comparable to the protein digestibility of *Spirulina* and *Chlorella* in catfish and tilapia (Sarker *et al.*, 2016; Teuling *et al.*, 2017) and cell ruptured *Chlorella* in juveniles of Atlantic salmon (*Salmo salar L)* (Tibbetts *et al.,* 2017*).* This indicated that they would be readily accessible for growth and hence suitable replacement for FM protein in *C. gariepinus* diet.

There is a great need to pay attention to lysine and methionine digestibility in the formulation of fish feed for all species of fish including catfish, using plant proteins. This is because they are important amino acids, which are limiting in terrestrial plants.

SP and CL diets in the current study showed ADCs 99.34% and 99.05% for lysine and 99.56 and 99.52% for methionine (Table 4.5). These values are comparable to lysine and methionine ADC values of 98.59% and 99.20% for this study reference diet (FM), 92.2- 97.9% and comparable to 95.5- 99.5 for soybean in channel catfish (Ambardekar *et al.*, 2009). Sarker *et al.* (2016) reported (> 80% and 90.0%) ADC lysine and methionine *Schizochytrium*, *Chlorella* and *Spirulina spp* Fed to tilapia. There is a similar

result reported by Dong *et al.* (2010) on hybrid tilapia Fed with corn gluten meal. Also, ADC values of 63.9% for methionine and 80.3% for lysine were observed in canola meal for hybrid tilapia (Zhou & Yue, 2012). There is a high ADC of methionine with the application of microalgal ingredients (Table 4.3), 99.51-99.56% comparable to the high ADC of methionine (94.6-97.4%) in sesame husk, cassava leaf meal, groundnut meal, soybean meal, meat and shrimp head meal reported for hybrid catfish and Nile tilapia (Guimaraes *et al.*, 2008; Tram *et al.*, 2011) and 100% for corn grain in channel catfish (Ambardekar *et al.*, 2009). All the essential amino acid was well digested in both *Spirulina* and *Chlorella* as their ADC were greater than 90.0%.

The lipid ADC of most fish species was reported to be within the range of 85 to 95% (NRC, 1993). Both protein and fat are mostly inside the algae cells, surrounded by cell walls. Consequently, protein and fat ADC can be used as an indication of differences *in vivo* nutrient accessibilities (Teuling *et al.*, 2017). In this study, the lipid ADC of both algae test diets (96.51 and 96.67%) differed significantly *($P < 0.05$)* compared to the reference diet (92.77%) and ADC of 88.4 and 85% for *Arthrospira* spp and *Chlorella* in *C.gariepinus* (Teuling *et al.*, 2017). It is, however, comparable to 94-96% for *Spirulina* and *Chlorella* in tilapia (Sarker *et al.*, 2016). The high ADC content of lipid from both test ingredients (Table 4.2) and diets (Table 4.5) showed that African catfish has a strong ability to use the microalgae's lipid component. Lipids are digestible nearly total in fish. Thus, they are a preferable source of energy than carbohydrates. Also, protein digestibility increases as dietary lipids increase (NRC, 1993). Protein and fat are assumed to be in no small extent hydrolysed and absorbed in the proximal and mid part of the intestine in African catfish (Harter *et al.*, 2015). *Chlorella* and *Spirulina* (ingredients) lipid and protein were digested 96-99% in the catfish. Seemingly, these nutrients were available to the digestive enzymes in the proximal part of the intestine before any cell wall fermentation that generally occurs at the distal part of the intestine

could take place. This may occur due to the combination of feed processing technique and stomach condition of the African catfish (which can reach pH 3.5 at 8 h after feeding) (Hlophe *et al.*, 2014; Teuling *et al.*, 2017). Specifically, feed processing and the abdominal condition can loosen the cellulose structure thereby allowing the nutrient inside the cell more accessible to hydrolysis. Protein has been found to release faster than cells were broken down indicating that cells need not be totally opened for the nutrients inside them to be accessible to the fish (Teuling *et al.*, 2017).

The composition, source, freshness of the source and processing temperature employed in producing the meal affect the ADC for energy. Arthrospira energy ADC values of 80-86% were reported in Arctic charr, Atlantic salmon, Nile tilapia and Caspian great sturgeon (Burr *et al.*, 2011; Safari *et al.*, 2016; Sarker *et al.*, 2016) and 84% for *Chlorella spp* (Sarker *et al.*, 2016).The greater than 90.0% energy digestibility observed in both test diets (Table 4.5), is similar to those obtained by earlier studies on *Spirulina* and *Chlorella* in other fish species and higher than 71-75 % for catfish (Teuling *et al.*, 2017). It is also higher when compared to values obtained for fishmeal, poultry-by-product and hydrolyzed feather meal in channel catfish (Kitagima & Fracalossi, 2011).

It seems that there is a relationship between the low dry matter and energy in some plant products, and the quantity and chemical composition of their carbohydrate (Dong *et al.*, 2010). High fibre content has been reported to affect the ADC of dietary energy and macronutrients by inhibiting the action of proteolytic enzymes and induction of amylase activity (Sklan *et al.*, 2004). Zhao *et al.* (2006) also reported a decrease in ADC dry matter, protein and energy with the increased addition of blue-green algae in gibel carp diets. In this study, ADC for lipid, dry matter and energy of both microalgae were greater than 85% (Table 4.2). The *Chlorella* used in this study were cultivated in closed

and sterilized bio-reactors with pure water hence they were soft and thin-celled and have their cell walls naturally broken by spray-drying. This may the reason for the high digestibility recorded in the experimental catfishes. Microalgal cell walls are not well understood, perhaps because of its complexity (Henry, 2012). The CL cell wall contains rigid wall components housed in an additional flexible polymeric medium, having three layers. The mid layer is thicker and has cellulose microfibrils while the outer one has polymerised carotenoid material which can prevent its nutrient absorption (Henry, 2012). However, *C. vulgaris* lack additional algaenan layer that is associated with other *Chlorella* species and cellulosic algae (Gerken *et al.*, 2013) which may be another reason for the high digestibility of the *C. vulgaris* used in the present study. *Spirulina* is known to possess peptidoglycan cell walls, mostly comprised of mucoproteins (Palinska & Krumbein, 2000) and therefore fish can digest it easily (Sarker *et al.*, 2016). However, Teuling *et al.* (2017) stated that the in vivo protein and fat digestibility of both algae and cyanobacteria (*Arthrospira*) in their study was not connected to the differences in mechanical cell wall hardness of both algae and cyanobacteria. Besides, cells need not be entirely opened for the nutrients inside them to be accessible to the fish. The high ADC of carbohydrate obtained for the *C. vulgaris* used in this study may also be ascribed to its broken cell wall leading to improved nutrient availability and absorption. This is supported by (Tibbetts *et al.*, 2017). Another factor is the pellet processing method. The pellets in this study where extruded before drying. Extrusion process has been reported to improve digestibility of ADC of DM and energy in *Nannochloropsis spp*, ash in defatted microalgae biomass and protein in *Desmodessmus spp* (Gong *et al.*, 2017). Additionally, since cellulose is likely to be the predominant component of the crude fibre fraction of *C. vulgaris* meals rather than hemicellulose lignin and pectin, pellet quality of the catfish feed may also be improved (Hansen & Storebakken, 2007; Li *et al.*, 2015; Tibbetts *et al.*, 2015)

Carbohydrate was well digested in both test ingredients (Table 4.2) and diets (Table 4.5) by experimental fish (ADCs 84-88 %). Fibre digestion is in accordance with what is obtainable in the literature (56.06-56.53%). Research has shown that African catfish can utilise fibre from terrestrial plants with an ADC value of 4- 56% (Leenhouwers *et al.*, 2007) depending on the type of fibre present. The ADC of carbohydrate obtained for the reference diets of this study was higher than findings of (Leenhouwers *et al.*, 2007; Teuling *et al.*, 2017). Tibbetts *et al.* (2017) on the other hand, recorded ADC of 35% and 43 % for carbohydrate and starch respectively with algae free (control) diet. As individual ingredients, CL and SP carbohydrate and fibre ADC values (Table 4.2) followed the same pattern as observed in both test diets. However, the *Chlorella* ADC (84.72%) value for CHO obtained in this study is in consistency with 84.6% reported for catfish (Teuling *et al.*, 2017).

Microalgae are recognized to be rich sources of n-3 LC-PUFA (Norambuena *et al.*, 2015). Short chain fatty acids showed lower digestibility than long-chain FAs. Austreng *et al.* (1980) reported the digestibility value of up to 100% of PUFAs like 20:5 or 22:6 acids in *Rainbow trout*. As seen in the Tables 4.4 and 4.7, all the FA fraction of the ingredients test and reference diets (SFA, MUFA, PUFA, n3 and n6 PUFA) were well digested (>95%) by the *C. gariepinus* juveniles. The PUFA fractions were the most digestible (97.51 - 99.01%) while MUFA was the lowest (96.08 - 97.41%). The ADC values of 99.07%, 99.64% and 99.13%, 99.68% obtained for EPA and DHA in *Spirulina* and *Chlorella* diets was higher than 98.45% and 98.79% for reference diet and within the range of 95.3- 100% reported for digestibility for whole cell and cell-ruptured *Chlorella* by juveniles of Atlantic salmon (*Salmo salar L*) (Tibbetts *et al.*, 2017). The two test diets showed improvement over the reference diet in the digestibility of the unsaturated fatty acid fractions. Perhaps is easier and more efficient to digest microalgae, which contain polar unit's phospholipids, than fish oil, which has triglycerides (neutral class lipids)

(Sarker *et al.*, 2016). Previous studies such as Teoh *et al.* (2011) have reported lower digestibility of fats in low phospholipid plants and fish oil. There is a dearth of studies on digestion of nutritional phospholipids, but the mechanisms of mammals and those of fish are believed to be similar generally (Sarker *et al.*, 2016). However, digestion and absorption of phospholipids and triglycerides in the small intestine occur in different ways.

Triglycerides are indissoluble in water; hence their digestion by enzymes and resultant absorption in the small intestine entails emulsification by bile salts through the formation of micelles. Bile does not digest phospholipids, but intestinal phospholipase A2, a secretion from the pancreas; which forms 1-acyl lysophospholipids and free FAs, absorbed by the cells of the intestinal mucosal (Henderson & Tocher, 1987; Sargent *et al.*, 2002). Lysophospholipids contain a detergent action; that help in the digestion of other lipids. This finding suggests that omega-3 phospholipids will be digested by *C. gariepinus* from both SP and CL with a higher efficacy than it will digest omega-3 triglycerols from fishmeal.

The highest ADCs of PUFA was observed in both test diets than the reference diet, which could be because of higher n-6 and n-3 PUFA, particularly the DHA contents in CL and SP, which it contains (Table 4.4). The results indicate that fish intestines digest LC-PUFA and PUFA better than SFA, and there is increase generally in ADCs of fatty acid profiles as the degree of unsaturation increases. This agrees with the findings of Bahurmiz and Ng (2007), Francis *et al.*(2007) and Sarker *et al.* (2016). Therefore, Olsen *et al*. (1998) reported that the digestive lipase in fish will digest fats in this order: HUFA → PUFA→ MUFA → SFA. Both algae diet had better MUFA and PUFA digestibility than the reference diet values (Table 4.7). The individual ingredients (SP and CL) and algae test diet (Tables 4.4 and 4.7) were both, however, had very high ADC (> 90%) of total n-6 and n-3 FAs. This finding support researchers such as Caballero *et al.* (2002) who have earlier

obtained ADCs between 92 and 99%. This study shows that the digestibility of unsaturated FAs was not affected in *C. gariepinus* by the rich lipid profiles of both algae.

5.2 Effects of partial replacements of fishmeal with *Spirulina platensis* and *Chlorella vulgaris* on growth performance and body composition of African catfish (*Clarias gariepinus*) fingerlings

After satisfying the gap of digestibility of both algae, the need arises to determine the growth performance of the catfish and the proximate body composition of the experimental fish. The study determined the effect of partial substitution of fishmeal by *Spirulina* or *Chlorella* at four graded levels each (12.5%, 25%, 50% and 75%) to assess the practicability of their use in *C. gariepinus* feed. The two microalgae varied in their chemical composition essential amino acid and fatty acid profiles. The results from this study suggest that both *Spirulina* and *Chlorella* have potentials to substitute fishmeal in *C. gariepinus* diets. The crude protein and lipid values of the experimental diets fall within the range recommended for optimal growth of catfish fingerlings by the FAO Fisheries Department (2009). As presented in Table 4.8 the diets were iso-nitrogenous (≈ 45%) and the lipid values ranged from 10.32 to 10.78%. These values are within the range recommended for optimal growth of catfish fingerlings by the FAO Fisheries Department (2009). Both *Chlorella* and *Spirulina* diets were good sources of amino acids (Table 3.5). The amounts of which were greater than values found by Jimoh *et al.* (2014) from sesame seed meal to catfish, and a slightly higher than values recommended levels for African catfish by Lovell (1989), Uys (1989) and Unprasert (1994) except for values obtained for methionine in CL75%, lysine in SP750 % and CL250 % whereas the values of methionine + cysteine and phenylalanine + tyrosine were reported by these authors, these values were recorded Separately. Cysteine was however not determined, as Lovell (1989) reported that non-EAAs like cysteine and tyrosine can replace about 60% and 50% catfish requirements for methionine and phenylalanine, respectively.

Fatty acids are imperative constituents of biomembranes of fish besides providing a premise of vitality (Parrish *et al.*, 2012). In many fish species, EPA and ARA are essential for vital metabolic functions (Tocher, 2015). Microalgae are the principal producers of FAs. Fish, for the most part, require linolenic acid (18:3n-3) for optimal growth. Catfish has the ability to change dietary linolenic acid to eicosapentaenoic acid (20:5n-3) which is adequate enough to meet its metabolic demands (Tucker & Hargreaves, 2004). Then-3 EFA prerequisite could be met by either 1% to 2% linolenic acid or 0.5% to 0.75% n-3 HUFAs (Satoh *et al.*, 1989) which can be supplied by supplementing marine fish oil, for example, menhaden oil in the diet (Morris *et al.*, 1995; Manning *et al.*, 2007). Fatty acids play vital roles in the development of catfish. The EFA requirements of *C. gariepinus* according to Uys and Hecht (1985) are linoleic acid (18:2n6), arachidonic (20:4n-6), linolenic (18:3n3), eicosapentaenoic (20:5n3), and docosahexaenoic (22:6n3) acids at proportion 1:1. The experimental diets contained either *Spirulina* or *Chlorella* supplemented with palm oil. The values for linoleic (9.51-19.91 % FA), linolenic (0.83-4.86 % FA), eicosapentaenoic (3.32 -7.84 % FA), and docosahexaenoic (2.39–27.72 % FA) acids obtained in the present study are higher than the recommendation for catfish by Satoh *et al.* (1989) and the ranges obtained by Li *et al.* (2009) from dried *Schizochytrium* sp. diets fed to channel catfish.

The outcome of this research shows that *C. gariepinus* fingerlings readily accepted the experimental diets as they exhibited good growth. The R^2 value of 0.9776 obtained further shows that replacing 75% of fishmeal with *Chlorella*, positively correlated with the growth of the fingerlings resulting in higher weight gain and SGR value (Table 4.8). This might be because of growth factor in *Chlorella* (Bengwayan *et al.*, 2010) while higher PER may be due to the high protein digestibility as observed in the digestibility study (Table 4.2). However, the higher weight gain can be ascribed to contribute to the reduced FCR in fingerlings Fed *Chlorella* diets, most especially 50 -

75% replacement. Although the *Chlorella* diets (50 and 75%) have higher CP, the high protein could induce the fish to use protein as an energy source and thus, the significant increase in weight gain observed. Surprisingly, the protein content of the diet is within the range recommended for catfish fingerlings growth. Optimum weight gain was achieved at 69.4% replacement of FM with *Chlorella* and higher substitution beyond this could not provide a further increase in weight gain. The previous study has shown that increasing the replacement level of fishmeal with *Chlorella spp* above 50% could not improve the growth performance of Nile Tilapia (Badwy *et al.*, 2008). Conversely, a study by Khani *et al.* (2017) has shown that optimum growth performance of Koi carp is best attained at 5% *C. vulgaris* replacement of fishmeal.

In addition, previous studies have attributed the comparable growth performance associated with *Chlorella* diets, to growth promoters such as sufficient amounts of macronutrients and *Chlorella* growth factors, present in *C. vulgaris* (Yamaguchi, 1996; Badwy *et al.*, 2008). More so, the growth performance associated with dietary *Chlorella* have been connected to high digestibility of the microalgae, and possession of significant concentration of polysaccharides, lipids, minerals and other bioactive compounds, which are involved in physiological activities (Xu *et al.*, 2014; Khani *et al.*, 2017). A study by Khani *et al.* (2017) suggested that *Chlorella* diets increase the digestive enzymes in the pancreas and intestine of Koi carp, thereby enhancing the diet utilization rate through an increase in the activity of digestive enzymes. Similarly, the inclusion of *Chlorella* up to 50% level in the diet of *Macrobrachium rosenbergii*, has been suggested to increase growth performance and level of digestive enzymatic activities (Radhakrishnan *et al.*, 2015). Another study by the author on the nutrient digestibility of dietary *Chlorella* showed that *Chlorella* was better digested and assimilated than the control (FM) diet (Tables 4.2 and 4.5).

The present study shows that *Spirulina* diets improve growth performances of *C. gariepinus* fingerlings, as indicated by the weight gain, FCR, PER and SGR. As the *Spirulina* inclusion level increases, the body weight gain of the fingerlings also increases, although this increase showed an indirect relationship with the CP of *Spirulina* diets. This implies that at 50% inclusion level showed the best FCR. The findings here also show that dietary *Spirulina* at the same inclusion level (50:50) with FM gives better PER, which could suggest that the diet is well digested and assimilated. It has been suggested that *S. platensis* improves the intestinal flora in fish, thereby allowing break down of ingestible feed components to extract more nutrients and stimulates the production of enzymes that transport fats for metabolisms instead of storage within the fish (James *et al.*, 2006). Abdel-Tawwab and Ahmad (2009) stated that the role *Spirulina* plays in the digestibility of nutrient and its ample contents of numerous vitamins and minerals is responsible for its positive effects on fish development.

The present study is inconsonant with the works of Palmegiano *et al.* (2005) and Nandeesha *et al.* (1998) who successfully replaced FM with up to 60 and 100 % dietary FM of *C. carpio* and Siberian sturgeon (*Acipenser baeri*) respectively, with no negative consequence.

Although some studies had recommended partial (0.5 – 5 %) replacement of fishmeal with *Spirulina* for optimal growth performance (Abdel-Tawwab & Ahmad, 2009; Promya & Chitmanat, 2011), but a total replacement (100 %) had also been reported to show improved growth performance. These studies, have also suggested that major inclusion (25 – 100%) of dietary *Spirulina* significantly increases the digestive enzymes activity of common carp, rohu and catla (Nandeesha *et al.*, 1994; Nandeesha *et al.*, 1998; Umesh *et al.*, 1994). More importantly, the cellular structure of *Spirulina* had been suggested to lack cellulose, thus, it is easily digestible (Promya & Chitmanat, 2011).

The R^2 (0.9468 and 0.9776) in *Spirulina* and *Chlorella* value obtained from the second order polynomial curve indicated that dietary *Spirulina* is positively correlated with the growth of the experimental fish and that 94.68% and 97.76% of the variation in weight of *Clarias gariepinus* can be explained by the replacement level of *Spirulina* and *Chlorella* respectively. However, a further increase in *Spirulina* may not retard growth, nor provide further improvement in *C. gariepinus* fingerlings as previously reported for catla (*Catla catla*) by Nandeesha *et al.* (2001). These variabilities obtained in growth reaction of fishes to dietary *Spirulina* and other algae species in the different studies visibly revealed that the growth response to algae may be species-specific. Other possible factors are variability of the nutrient composition, inclusion level of *Spirulina* or other algae as well as the nutritional contents of the test diets to which *Spirulina* is contained within (Nandeesha *et al.*, 1998; Olvera-Novoa *et al.*, 1998; Nandeesha *et al.*, 2001; Takeuchi *et al.*, 2002). Olvera-Novoa *et al.* (1998) suggested that harmful effect connected with the high addition of *Spirulina* in the diet on fish growth may be attributable to declines in phosphorous accessibility and reduction in feed palatability.

Survival rates were more than 85% in all the groups, signifying that mortality was not diet related.

Fulctons (K) factor provides reliable information on the actual state of health, growth, and well-being of fish (Araneda *et al.*, 2008). The present study reveals that both *Chlorella* and *Spirulina* diets showed better Fulton's condition factor and survival rate when compared to the fish Fed control diet. This further corroborates the high growth performance exhibited by fish Fed the alga diets, and hence, it can be suggested that these factors put together signify good health condition of the fish. It is also noteworthy that dietary *Chlorella* and *Spirulina* have been suggested to improve the immune system of fish (Güroy *et al.*, 2011; Xu *et al.*, 2014; Khani *et al.*, 2017).

The results obtained in this study showed that there is a positive correlation between the dietary protein and the fish muscle CP. The whole-body composition of *C. gariepinus* Fed dietary *Chlorella* and *Spirulina* shows higher CP than the control. This can be connected to the higher protein productive values observed with the alga diets, which signify good assimilation of the protein. It has been suggested that fish do not have a specific protein requirement but rather, a definite requirement for essential amino acids (EAA) content of the protein (Miles & Chapman, 2007). This is because the dietary proteins will be digested and broken down to amino acids, which can be used efficiently for maintenance, health and synthesis of worn out tissues, and thus, result in maximum feed efficiency and growth (Miles & Chapman, 2007). This assertion can be supported by the profiles of EAA exhibited by the alga diets as observed in the present study.

In this work, protein decreased as the *Spirulina* increases while fat increases in a dose-dependent manner. The high fat and energy in some of the carcasses may be because of the varied inclusion level of the palm oil, which was mixed with the fish oil. This corroborates the findings of researchers who opined that when plant protein are included in the diet of fish, there is a propensity for an increase in the fish lipid (Yildirim-Aksoy *et al.*, 2007; Bake *et al.*, 2016). The findings in this study are consistent with other findings which found *Spirulina* to increase fat deposition (Atack *et al.*, 1979; Watanabe *et al.*, 1990; Nandeesha *et al.*, 1998; Nandeesha *et al.*, 2001). However, it differed from that of *C. carpio* where a significant decrease occurred in lipid body composition by similar *Spirulina* administration. This was because of the effects of different *Spirulina* species on fat deposition (Nandeesha *et al.*, 1998; Nandeesha *et al.*, 2001). Abdel-Tawwab and Ahmad (2009) stated that the influence of *Spirulina* on body lipid as well as protein are linked to their production plus build-up level, as well as the growth rate of the animal.

Chlorella fed fish shows decreases in flesh fat deposit with its increase in the diets, this may be owing to the high quantity of polyunsaturated fatty acid in the *Chlorella* in this study or due to the presence β-1,3-glucan a strong immune booster, free-radical forager, and reducer of blood fat. *Chlorella* had superior activity in hindering lipid peroxidation compared to glutathione and has antioxidant properties (Bengwayan *et al.*, 2010). Amano and Noda (1985) also reported a reduction in fat levels in the flesh and liver of Ayu by feeding 2% *Chlorella* extract. Also, dietary lipid tends to decrease with increasing *Chlorella* (Table 3.4), the SP75 % diet had the highest lipid (10.78 %) and CL75% the lowest (10.32%). This agrees with Nandeesha *et al.* (1998) and Abdulrahman and Ameen (2013) for *Spirulina* and in contrast with the findings of Badwy *et al.* (2008) who observed an increase in dietary fat with increased *Chlorella* and *Scenedesmus* supplementation in their diets.

Ash was higher in *Chlorella* than *Spirulina* diets in this study (Table 3.4) while Badwy *et al.* (2008) also documented the highest ash in Nile tilapia fed 75% of *Chlorella* and *Scenedesmus*. Initial fishes had significantly higher ash in their tissues while SP75% had the lowest (Table 4.9). This follows the trend and supports the observation that ash tends to decrease in both fish flesh and experimental diets with a dietary increase in *Spirulina*. Olvera-Novoa *et al.* (1998) observed the highest and lowest ash value in the control and 80% *Spirulina* diet of *Tilapia mozambique* respectively. On the contrary, Hossein *et al.* (2013) recorded an increase in carcass ash and low lipid deposition in fish with increased *Spirulina* and Alga100 supplementation in diets.

Flesh moisture was observed to inversely correlate to flesh lipid in both algae supplementation (Table 4.9). This is contrary to the observations in *Azolla* that led to a significant increase in ash and moisture with its increase supplementation in the diets of Nile tilapia (Ibrahim *et al.,* 2007).

In this study, this study *Chlorella* feed had highest NFE than the *Spirulina* and control feed in a dose-dependent manner with the highest 22.28% in the CL75% feed. This might be the reason for the slightly low feed consumption in these groups although insignificant as it does not negatively affect their growth. On the contrary, the initial flesh had significantly ($P < 0.05$) greater NFE than other groups. *Spirulina* feeds had the highest fibre than other groups, while the proximate composition of the muscles of all the fish in this study were less than 0.1% in fibre content hence it was not documented.

There was a positive correlation between energy in the carcass and dietary lipid, as SP75% fish had a significantly higher lipid (10.52%) and energy (473.96Kcal /100 g) in the flesh. Lipids are important sources of energy (Webster & Lim, 2002), as they have more energy per unit weight than any organic compound, one gram of fat comprises about twofold as much aggregate energy as a single gram of protein or carbohydrate (Guillaume, 2001). Previous findings have disclosed that considerable use of vegetable oil as an energy source in fish diets produced an encouraging growth reaction in fish (Babalola & Adebayo, 2007; Aderolu & Akinremi, 2009). Lim *et al.* (2001) and Ng *et al.* (2000), stated that up to 8% and 90% of nutritional fish oil could be substituted with refined, bleached, and deodorized palm oleum or crude palm oil devoid of negative consequence on growth or feed use of African catfish and green catfish (*Mystus nemurus*) respectively. Sotolu (2010) documented a similar observation by feeding diets comprising soybean, benniseed, groundnut, and palm oils to *C. gariepinus* fingerlings.

In this study, fish oil and palm oil at ratio 1:3 were used and the results obtained have demonstrated that they are of good nutrient composition and had no palatability problem and utilization was adequate, although may be responsible for carcass fat build up. Previously, the inclusion of vegetable oils in the diet of seabream was also found to promote fat build-up in the liver (Caballero *et al.*, 2004). However, there seems not to be

a direct relationship between energy in the carcass and diets. This implies that energy in the carcass might have been derived from different components of the diets, mostly lipids.

5.3 Dietary effects of *Spirulina* and *Chlorella* on growth and haemato-immunological response of African catfish to pathogenic *Aeromonas hydrophila*

Research has shown that the general health and immune responses of fish are strongly related to their nutrition (Priya *et al.*, 2004; Kumari & Sahoo, 2005). Consequently, the current study evaluated the effects of partial substitution of fishmeal (being the source of protein in aquafeed) with *Spirulina* and *Chlorella* on growth and immunity of *C. gariepinus* juveniles. Although few studies (Promya & Chitmanat, 2011; Duncan & Klesius, 1996) have studied the effect of *Spirulina* on growth and innate immunity of *C. gariepinus* and channel catfish, there is a dearth of information on the effect of *Chlorella* on catfish haemato-biochemical activities and immunity. Therefore, a preliminary comparative study was carried out on all the trial diets. The outcomes of which demonstrated that the protein contents of the experimental diets as shown in Table 3.7 were within the range of 35 $g100g^{-1}$ (Ali, 2001) and 38 $g100g^{-1}$ (Uys, 1989) recommended for catfish growth. All the fishes readily consumed the feed and converted to flesh as indicated by the increase in weight gain, SGR, PER, PI and a correspondingly low FCR (Table 4.10). Dietary *Chlorella* at 50% inclusion levels significantly enhanced the growth parameters of African catfish except for PI, which though was numerically higher in the diet, was insignificant. Nevertheless, except for FCR and PER, there was no significant difference between CL50% and SP50%. The growth improvement of *Spirulina* in this study may be because of its high digestibility of nutrients especially protein and lipid as well as amino and fatty acids observed in the digestibility study reported in Section 4.1.7 to 4.1.9 of this thesis. *Spirulina* has been linked with high

digestibility that is triggered by high digestive enzymes activity, the breakdown of indigestible components, vitamins production and intestinal microbiota leading to increased appetite and improved feed intake (Nandeesha *et al.*, 1998; Abdel-Tawwab & Ahmad, 2009; Dawood *et al.*, 2014; Dawood *et al.*, 2016). *Spirulina* is also linked with increased ability to absorb nutrients (Promya & Chitmanat, 2011) On the other hand, the growth stimulating effects of the *Chlorella* (*C. vulgaris*) in this study, may be due to the type (thin and broken cell wall) which makes it highly digestible and the processing method (spray- drying by pressure release) that conserved most of the nutrients within the algae may be responsible for the improved growth of the *Chlorella* fed fish. The *Chlorella* growth factor (CGF) in *Chlorella* has also been associated with its growth stimulating effect (Bengwayan *et al.*, 2010). On the other hand, dietary *Chlorella* has been found to promote the activity of the digestive enzyme in the hepatopancreas leading to increasing diet utilisation and growth of gibel carp (Xu *et al.*, 2014).

The physiological status of the experimented catfish was investigated using haematological parameters. These parameters are also suitable for assessment of nutritional status and feed composition relative to the environmental conditions that affect the fish (Svobodová *et al.*, 2005).

Blood and biochemistry parameters remain indispensable traits designed for measuring the health and physical status of an organism. They mirror the physiological reaction of an animal to its inner environment, which may be due to numerous factors, including feed and feeding (Etim *et al.*, 2013), age (Daramola *et al.*, 2005), environment, sex, stress, pathological (Gabriel *et al.*, 2004) and toxicological factors (Velisek *et al.*, 2013; Gadhave *et al.*, 2014). It also makes available valuable data on the resilient status of the animal (Daramola *et al.*, 2005). Any alteration in the usual haematological and biochemical blood profile of the fish reflects its condition (Myers & McGavin, 2007).

Therefore, determination of these parameters gives valued data concerning physical changes and the efficient status of essential organs like liver, heart, kidney and pancreas, and can be used to predict the degree of organ damage in the fish body (Benjamin, 1978; Coles, 1986).

Red blood cells (RBC) are the major reliable pointers of various stressors. Disparity or deficiencies of essential minerals, fatty acids, vitamins and other important macromolecules have been known to decrease RBC counts, HCT and HBG in fish (Wedemeyer *et al.*, 1983; Schreck, 1996; Sarkar *et al.*, 1999). HCT values are more sensitive indicators of the immediate vitamin situation and could, therefore, be used to envisage future growth performance (Barrows *et al.*, 2008). In this study, all the pre-challenged RBC, HCT and HGB values were high and stayed within or slightly higher than the limits stated by Okorie-Kanu and Unakalamba (2015) which were RBC (2.33-2.69) Hgb (10.34 -12.41) and Hct (30-44) and RBC (3.10) reported by (Al-Dohail *et al.*, 2009) for *C. gariepinus* fish, suggesting that all the experimental diets do not have undesirable consequences on the percentage of the total erythrocytes HCT and HGB levels, as well as the oxygen-carrying ability of the blood. Increased RBC and HGB in fish have been linked to its growth and ability to boost its activity to meet occasional demands (Ayoola *et al.*, 2013). The more active fishes like *C. gariepinus* tend to have higher haemoglobin than the inactive ones. This increase in haematological (Hgb, Hct and RBC) parameters in a dose-dependent manner detected in fish fed both *Spirulina* and *Chlorella* is indicative of a better health status. *Spirulina* and *Chlorella* have been reported to be rich in iron (Yeganeh *et al.*, 2015) and folic acid (Radhakrishnan *et al.*, 2014; Khani *et al.*, 2017) that was found to have a substantial effect on erythropoiesis through the increase of RBC and haemoglobin counts (Kapoor & Mehta, 1992). On the other hand, since RBC has been found to be one of the major production sites for free radicals (Babak *et al.*, 2012), feeding *Spirulina* and *Chlorella* that are rich sources of

bioactive and natural antioxidants like vitamins C and E, carotenoids and phycocyanin, can deter degeneration of RBC (Radhakrishnan *et al.*, 2014; Yeganeh *et al.*, 2015). This support the works of Al-Dohail *et al.* (2009) and Ayoola *et al.* (2013) on African catfish fed *L. acidophilus* and *Moringa oleifera, Labeo rohita* fingerlings and rainbow trout fed *Spirulina*, and Koi carp fed *Chlorella vulgaris* (Andrews *et al.*, 2011; Yeganeh *et al.*, 2015; Khani *et al.*, 2017). The reduction in these parameters post-challenge was because of anaemia and haemo-dilution triggered by impaired osmoregulation, induced these by the presence of *A. hydrophila* infection (Bittencourt *et al.*, 2003). A similar observation was reported by Ranzani-Paiva *et al.* (2004), Misra *et al.* (2006) and Kumar *et al.* (2007), who also recorded reduced erythrocytes, RBC and HGB in Nile tilapia and *L. rohita* juveniles post-*A.hydrophila* challenge. Similarly, Foda (1973) also discovered a decrease of haemoglobin in Atlantic salmon caused by furunculosis, triggered by *A. salmonicida*. The treated groups were, however, better than control in before and after-challenge as they exhibited significantly higher values than control in RBC, Hgb and Hct. This is due to the modulatory effects of both algae. The modulatory effect of *Spirulina* has been attributed to its phycocyanin content (Yeganeh *et al.*, 2015) while the β-1,3-glucan in *Chlorella* is known for its potent immune-stimulatory effect, blood lipids reduction and free-radicals scavenging property (Spolaore *et al.*, 2006; Xu *et al.*, 2014). This supports the findings of Harikrishnan *et al.* (2003) who recorded an elevated Hgb and erythrocyte level as a result of treating *C. carpio* infected with *A. hydrophila* and Kumar *et al.* (2013) Argulus-infested goldfish (*Carassius auratus* Lin 1758) with *Azadirachta indica*.

RBC parameters are employed to estimate the dimensions and haemoglobin contents of the RBCs (Brown, 1993). The MCV (115-117 fl), MCH 30.28-30.55) and MCHC (25.88-26.73) pre-challenge were within the normal of 100-116.2 (Sayed & Fawzy, 2014), 28.05- 32.67 (Okorie-Kanu & Unakalamba, 2015) and slightly higher than 22.41-25.88 (Sayed & Fawzy, 2014) ranges in *C. gariepinus*. There was, however, a

noteworthy change in these indices post-challenge. The rise in MCV might be ascribed to the protuberance of the erythrocytes, leading to macrocytic anaemia in response to stress caused by *A. hydrophila.* This leads to reduced ATP and increased oxygen affinity of the blood (Soivio & Nikinmaa, 1981; Bhagwant & Bhikajee, 2000). The MCV values of algae-treated fishes were significantly lower than control post-challenge. On the other hand, the fluctuations in the MCH and MCHC post- *A. hydrophila challenge* signified that the concentration of haemoglobin in the RBC was much lower in the post-challenged fish compared to the healthy fish due to the anaemic condition. This finding agrees with that of Kumar *et al.* (2013) in the case of Argulus-infested goldfish and Hedayati and Ghaffari (2013) in Silver carp exposed to mercury chloride.

Increase in platelets count in fish is a sign of differential thrombocytosis, meaning there is an upsurge in the stimulation of platelets meant for good clotting during injury or tissue damage (Etim *et al.*, 1999). In this study, there was a substantial increase in platelets post-challenge with CL50% as compared to control and other treated groups. All the treated diets were higher than control, an indication that both *Chlorella* and *Spirulina* diets stimulate good coagulation during injury. A similar observation was documented by Sayed and Fawzy (2014) in *C. gariepinus* with increased dietary *Spirulina* levels.

The white blood cell, red blood cell, and haematocrit (Hct) are employed in checking feed toxicity and fish health (Ozovehe, 2013). White blood cells (WBC) are cells of the immune system which help defend the body from infectious diseases and foreign organisms (MedlinePlus National Institute of Health, 2012). A high WBC count is an indication of stress, inflammation, infection, immune system disorders, anaemia, bone marrow tumour and tissue damage, among others (Rodelo *et al.*, 2012; Valencia, 2012; Braun, 2013), while a low WBC count may be due to liver or spleen diseases, scarring, bone marrow deficiency or failure (Etim *et al.*, 2013). White blood cells perform

a vital role in non-specific immunity and are a pointer to fish well-being. An increase in WBC count decreases the immunosuppression caused by *A. hydrophila* due to the presence of ß glucans, polysaccharides and chlorophyll ingredients of significant bioactivity, which have *in vitro* and *in vivo* immunostimulants activities. This result is in accordance with the findings which demonstrated an upsurge in WBC counts in *Labeo rohita* juveniles after feeding with 1% yeast, levamisole and ascorbic acid (Choudhury *et al.*, 2005; Andrews *et al.*, 2011), garlic peels in catfish (Thanikachalam *et al.*, 2010) and *Spirulina* in *C. gariepinus* and rainbow trout (Promya & Chitmanat, 2011; Sayed & Fawzy, 2014; Yeganeh *et al.*, 2015).

Maheswaran *et al.* (2008) reported an increased number of WBC, reduction of RBC, and reduced HGB in *C. gariepinus* subjected to different levels of mercury oxide for 35 days. The increase in WBC observed in this study after *A. hydrophila* exposure, irrespective of source and inclusion levels of dietary immunostimulants, might be ascribed to a stimulation of the immune system in response to tissue injury triggered by *A. hydrophila* infection. Fishes Fed 75% *Spirulina*, CL50% and other algae treated groups showed higher RBC, HCT, HGB (pre and post-challenge), than control irrespective of the condition of the fish. This is like the findings of Hrubec *et al.* (2000) and Promya and Chitmanat (2011) in Tilapia and *C. gariepinus* respectively. This increase may be ascribed to the existence of C-phycocyanin, β-Carotene, sulphated polysaccharides in *Spirulina*, which helps in building the immune capacity (Vonshak, 1997; Wu *et al.*, 2016) and carotenoids, vitamins and glucans in *Chlorella* (Khani *et al.*, 2017) which perform the specific function of boosting in addition to deepening the ability of the fish to resist infections by means of stress diminution (Nakono *et al.*, 2003). However, the significant post-challenge increase of WBC observed in the zero algae (control) group, may be attributed to lower immunity which translated to higher mortality (80%) as seen in Figure 4.3.

Fish biochemistry profiles are valuable and reliable indicators of the general well-being of fish (Dawood *et al.*, 2016). Many studies have related differences in biochemical values with living environments and age (Satheeshkumar *et al.*, 2012). In the current study, all the biochemical parameters remained within the range (99-117) reported by Myburgh *et al.* (2008) for *C. gariepinus.* Under stressful conditions, fish will exhibit an increased plasma cortisol followed by a corresponding increase in plasma glucose levels (Soltanian *et al.*, 2014). In the current study, plasma glucose ranged from 72.45 -78.09 mg/dl and were slightly lower than the values of Al-Dohail *et al.* (2009), which is 78.94 for catfish fed *Lactobacillus acidophilus*, meaning that prolonged feeding with both *Spirulina* and *Chlorella* was not stressful to the fish. The glucose level was also found to decrease with increased supplementation of both algae pre- and post- bacterial challenge. Glucose concentration is usually taken as a stress indicator in fish over adrenaline or cortisol because during a stressful condition, higher blood glucose is maintained generally through glycogenesis (David *et al.*, 2005). Therefore, the substantial variation between the pre- and post-challenge glucose may be due to the injection of *A. hydrophila* which triggered a remarkable upsurge in plasma glucose (due to increased energy requirement to neutralise the toxin secreted by the bacteria) in all the experimental fish, with the control set having significantly higher glucose than all the treated groups' pre- and post-challenge. These findings support the results of Kaleeswaran *et al.* (2012) on *Catla catla*-fed *Cynodon dactylon* post-*A.hydrophila* challenge.

Blood and serum total protein concentration is normally used as a basic index of general biological well-being (Svetina *et al.*, 2002). Serum protein, especially albumin and globulin, play a key role in maintaining immune reaction of fish, as their increase is believed to relate to stronger innate immune response in fishes (Jha *et al.*, 2007; Acharya & Mohanty, 2014). Fish fed different immunostimulants were always found to be higher in serum albumins and proteins and are believed to be linked with a robust innate immune

response in these fishes (Kaleeswaran *et al.*, 2012). Albumin and globulins help to sustain the osmotic pressure to maintain a healthy immune system and serve as a plasma carrier (Nya & Austin, 2009). An elevated plasma protein, albumin and globulin in a dose-dependent manner of the fish fed with both algae imply a strong immune response of the fed fish. This corroborates with the report of Yeganeh *et al.* (2015) on *Spirulina*. The albumin accounts for 55% of the total protein and transport lipids and steroid hormones in the fish blood (Acharya & Mohanty, 2014; Fedonenko *et al.*, 2016). Albumin-globulin ratio helps in the evaluation of different kidney and liver diseases. The Gammaglobulin fraction is the basis of all the immunologically effective protein of the blood, therefore the lower albumin-globulin ratio in all the algae fed groups signifies that more amounts of globulin are present in the blood and thus an indication of higher resistance to infection (Andrews *et al.*, 2011). The increase in WBC count, percentage survival and NBT values in the algae treated fishes supported this result. This is in consonance with Andrews *et al.* (2011), Kim *et al.* (2013), Xu *et al.* (2014) and Yeganeh *et al.* (2015), who reported an increased plasma protein and globulin in rainbow trout, olive flounder-fed *Spirulina* and gibel carp-fed *Chlorella*. Alterations in serum albumin and globulin affect the level of total protein (Shahsavani *et al.*, 2010). The post-challenge decrease in both serum protein, albumin and globulin may be due to loss of vascular serum protein due to higher permeability (Green, 1978; Ellis *et al.*, 1981), decreased synthesis, and non-specific proteolysis of serum protein (Ellis, 1981; Misra *et al.*, 2006). Conversely, the alterations in the albumin-globulin (A/G) ratio observed in the experimental fish post challenge, maybe as a result of a poorer liver function or protein loss owing to reduced gill function (Omitoyin, 2007). Consequently, the lower A/G ratio detected pre-and post-challenge especially in the algae treated groups might be due to reduced synthesis of albumin and increased production of globulin to cope with the immunological functions leading to improved immune response.

Alterations in enzyme profiles remain important toxicity guides (biomarkers) that have been widely used in evaluating the biochemical and functional well-being of vital organs in fish. ALT and AST activities are important pointers of the liver and kidney function in fish (Coz-Rakovac *et al.*, 2005). Increased serum ALT and AST have been shown to be associated with cellular damage, increased membrane absorbency, and intercellular metabolism when the physical integrity of the liver is damaged. Pan *et al.* (2003) discovered a rise in serum ALT and AST when hepatopancreatic complexes of shrimp were ruptured. Organic stress and a higher concentration of biological matter have a damaging effect on the liver tissues. Venkateswara Rao (2006) reported an increase in ALT, AST and ALP levels in tilapia exposed to organophosphorus insecticide (RPRP11). Kumar *et al.* (2013) also made a similar observation in *Argulus* infested goldfish. In this index research, the levels of AST, ALT and ALP increased post *A. hydrophila* challenge. This increase may be associated with the increase in the generation of reactive oxygen species (ROS) in response to *A. hydrophila* bacteria (Banaee *et al.*, 2017). However, their levels were shown to be decreased in the treatment groups as compared to control, irrespective of pre- and post-challenge status. This is in conformity with the findings of Vasudeva Rao *et al.* (2006) in *L. rohita* fed a diet containing herb *Achyranthes aspera* after *A. hydrophila* exposure and in Argulus infested goldfish treated with azadirachtin (Kumar *et al.*, 2013). ALP is an enzyme that transports glycogen, synthesizes protein and certain enzymes and regulates secretary activities (Pradhan & Das, 2015). It also facilitates the transport of membranes and splits various phosphorus esterases at an alkaline Ph. Therefore, any changes in ALP activity may affect the animal in a different way. Reduction in serum/tissues ALP and acid phosphatase activities in carp due to stress induced by the sublethal effect of nitrite was reported by Das *et al.*(2009). Increased ALP activity was recorded in *Rohu* fed with *Chlorella* from 60-100 days and turmeric for 60 days post *A. hydrophila* challenge (Sahu *et al.*, 2008; Pradhan & Das, 2015) This is in

consonance with the observation of this study as the ALP (except for the control which decreases) increased significantly post *A. hydrophila* challenge especially in *Chlorella* (CL75%) treated group. Intensification of phosphatase signifies a higher breakdown of energy reserves that is normally used for fish growth and survival (Pradhan & Das, 2015). Serum creatinine is used to measure by-products of muscle metabolism that is excreted unchanged by the kidneys. A rise of its level is an indication of kidney failure while a decrease may be as a result gastrointestinal bleeding, dehydration, starvation or urinary tract obstruction (Ajeniyi & Solomon, 2014). The low level of creatinine recorded post challenge may be ascribed to these. Inyang *et al.* (2010) recorded similar findings when *C. gariepinus* was exposed to the sublethal effect of diazinon. This result is supported by the discoveries of Pradhan and Das (2015), who stated that feeding 0.1 – 1.0g kg^{-1} of dietary *Chlorella vulgaris* contributed positively to the growth with no negative effect on the liver function of rohu even after *Aeromonas hydrophila* challenge. Kim *et al.* (2013) also made a similar observation by feeding *Spirulina* to Olive flounder. This shows the hepato-preservative properties of dietary *Spirulina* and *Chlorella* which may be associated with their antioxidant and free radical scavenging properties against damage caused by *A. hydrophila* and consequently preserving the physical integrity of the cell membranes of the liver.

Spirulina and *Chlorella* have been reported to have hypocholesterolemic effects (Khan *et al.*, 2005; Peiretti & Meineri, 2011; Kim *et al.*, 2013). Such an effect has either remained un-investigated in *C. gariepinus*, or there is a dearth of information in the literature. In this study, the effects of both algae on serum cholesterol (triglyceride, total, HDL, and LDL) levels were examined. Significant reductions in triglyceride total and LDL cholesterol (with increased algae supplementation), and a significant intensification in HDL cholesterol were observed in *Spirulina* and *Chlorella* when compared to the control group. This agrees with the findings of Kim *et al.* (2002), Kim *et al.* (2013), Xu

et al. (2014) and Yeganeh *et al.* (2015) on olive flounder-fed *Chlorella* and *Spirulina* and gibel carp-fed *Chlorella* respectively. Research has shown that LDL transports cholesterol from the peripheral tissues to the liver, while HDL returns the cholesterol produced and/or deposited inside the peripheral cells to the liver for re-use, thus, providing protection against atherosclerosis development. Accordingly, the increase in the HDL cholesterol observed in this study, suggests that both algae can improve the fish cardiovascular activity of *Chlorella*. *Spirulina* and *Chlorella* are natural sources of antioxidants like phenolic compounds, phycocyanin, β-carotene (Colla *et al.*, 2008), polyunsaturated fatty acids (Yeganeh *et al.*, 2015) and glucans especially β-1,3-glucan in *Chlorella* (Spolaore *et al.*, 2006) which have therapeutic properties and reduce blood lipids. A general decreasing trend of triglyceride and serum cholesterol except for LDL (which increased) was also observed post-challenge. This finding is supported by the work of Kumar *et al.* (2007) on *L. rohita* challenged with *A. hydrophila.*

Fish are most vulnerable to bacteria and viruses' due to their direct contact with the aquatic environment. There was no mortality throughout the 16 week-experiment pre-*A. hydrophila* challenge. However, 24 hours post-challenge, all fish developed clinical signs of the greyish-white lesion on the surface of the body and caudal fin, loss of balance, redness of the fin and loss of appetite, followed by mortality as early as 48 hours post-challenge. The highest mortality of 80% (Figure 4.3) was recorded in the control while the lowest (26.67%) was recorded in the CL50% group. This confirms that *A. hydrophila* was pathogenic to *C. gariepinus* as earlier observed by (Taufek, 2016). The lower mortality and quick wound recovery observed in both algae-treated group as equated with the control group suggested the acquisition of non-specific immunity due to feeding of both *Spirulina* and *Chlorella* (Abdel-Tawwab & Ahmad, 2009; Andrews *et al.*, 2011; Promya & Chitmanat, 2011; Kim *et al.*, 2013; Qihuan *et al.*, 2014; Pradhan & Das, 2015).

To confirm pathogenicity of *A. hydrophila,* the intestine and liver of dead fishes were cultured to isolate the bacteria. The highest bacteria load (1.29×10^5 and 1.07×10^5 cfu ml^{-1}) was detected in the intestine and liver of the control group, while the lowest intestinal and liver load was recorded in CL50%. This corresponds to the mortality trend (Figure 4.3). There were no significant changes in the bacteria load in the intestine of CL50% and SP75% fishes. The intestinal bacterium load was, however, higher than that of the liver irrespective of the dietary effects. A similar observation was made in perch with 9.4×10^8 cfu g^{-1} and 2.9×10^6 cfu g^{-1} in the intestine and liver respectively (Hossain *et al.*, 2013). Mostafa *et al.* (2008) also recorded 1.8×10^9 in the intestine as against liver's 6.46×10^8 g^{-1} in the Asian stinging catfish. On the contrary, Sarkar and Rashid (2012) recorded higher bacteria load of 6.5×10^8 and lowest of 5.6×10^7 cfu/g in the liver and intestine of *C. batrachus* respectively.

Fish, unlike mammals, frequently rely on innate immunity (Swain *et al.*, 2007). Therefore, substantial care has been paid to the use of bioactive ingredients to activate natural protection. Many components and bacteriological concoctions assist phagocyte stimulation via a corresponding rise in the production of generalized defence cells and antibodies (Chung & Secombes, 1987; Caruso *et al.*, 2002). The C-phycocyanin in *Spirulina*, carotenoids, vitamins and other bioactive materials in *Chlorella* has been linked to their immunomodulatory activity (Venkataraman, 1997; Niu *et al.*, 2007; Khani *et al.*, 2017).

During phagocytosis, stimulation of the cell membrane initiates the production of oxygen-free radicals by producing superoxide anion (O_2^-) and its derivatives such as hydrogen peroxide (H_2O_2) and hydroxyl-free radicals (OH) in a procedure recognized as respiratory burst activity (RBA). These volatile O_2 intermediates have been observed to possess robust bactericidal actions against fish pathogens (Hardie *et al.*, 1996; Itou *et al.*,

1997). The RBA of phagocytes was assessed in this research by a reduction of nitro blue tetrazolium (NBT) by intracellular superoxide radicals generated by leukocytes. A substantial value of NBT was observed in both *Chlorella* and *Spirulina* diets than in control, with the topmost activity observed in the CL50% set, signifying maximum enhancement of immunity of *C. gariepinus* fish. This is in harmony with findings of earlier researchers on Nile tilapia, *L. rohita*, olive flounder and *C. carpio* respectively (Kumar *et al.*, 2005; Watanuki *et al.*, 2006; Abdel-Tawwab & Ahmad, 2009; Andrews *et al.*, 2011; Kim *et al.*, 2013).

Lysozymes are found in mucus, ova and serum of fish, and perform a vital mediatory defensive function in innate immunity by breaching the cell wall of bacteria and proceeding to trigger the phagocytosis of bacteria (Murray & Fletcher, 1976; Saurabh & Sahoo, 2010). Fish serum lysozyme activity is understood to emanate from leukocytic roots (Lie *et al.*, 1989; Cecchini *et al.*, 2000). Significantly higher day 10 and 18 lysozyme activities were noticed for both *Chlorella* and *Spirulina* supplementation levels, with the highest day 10 and 18 activities detected (128.67 and 145.00 Uml^{-1}) in CL75%. This suggests that *Chlorella* may contain some bioactive ingredients that are involved in the regulating of the immune response of the fish fed with it. This is in harmony with the observations in Atlantic salmon and *C. carpio* (Siwicki & Studnicka, 1987; Møyner *et al.*, 1993), which exhibited higher serum lysozyme activities post-challenged with *Aeromonas salmonicida* and *punctata* respectively. Promya and Chitmanat (2011) and Kim *et al.* (2013) disclosed a significant surge in *C. gariepinus* and Olive flounder-Fed 3% and 5% dietary *Spirulina* and *Spirulina* 6.8 + quercetin respectively. The substantial rise in the leukocyte levels after *A. hydrophila* challenge portends the boosting of the non-specific immune system of the algae treated fish. A rise in the c-type lysozyme m-RNA in head, kidney, ovary and spleen of Japanese flounder infected with *Edwardsiella tarda* suggested the intensification of lysozyme synthesis (Hikima *et al.*, 1997), which

corroborates the findings of this study. Zhang *et al.* (2014) revealed that dietary *Chlorella* can increase fish lysozyme and SOD and contains bioactive materials that can regulate fish innate and adaptive immunity, by increasing the levels of immunoglobulin M and D, interleukin-22 and chemokine (C-C motif) ligand 5 in some tissues, as well as improving the C4 complement levels (Khani *et al.*, 2017).

Sakai (1999) reported that the efficacy of oral and immersion methods of administration of immunostimulants decreases with the long-term administration and that overdosage induces immunosuppression in fish. This study supports the observation of Henry (2012), who stated that while plants are important to terrestrial animals, microalgae represent the natural biological process base, as well as the first supply of all the phytonutrients within the aquatic organic phenomenon (Henry, 2012). They are a characteristic part of the diet of several larval fish, either eaten directly or attained from the gut contents of prey species, for example, copepods and rotifers (Henry, 2012). They are also reported to improve the immune and digestive functions of larvae (Cahu *et al.*, 1998; Spolaore *et al.*, 2006).

To support these statements, this research work replaced up to 75% of FM (18.75 % of the total diet) as compared to previous workers who used 5% *Spirulina* on catfish (Promya & Chitmanat, 2011) and 0.4-2% *Chlorella* on gibel fish (Xu *et al.*, 2014) as mere additives. Although there are limited reports (for comparison) regarding the use of SP and CL as replacements in the diets of *C. gariepinus*, findings from this study suggested that the fish immune system was not compromised with the use of the algae. This can be seen from the rich amino and fatty acid profile of the SP and CL, the improvement in haemato-biochemical parameters (pre-and post *A. hydrophila* challenge), RBA, lysosome activity, survivability in the treated fishes. Besides, as concentrations of harmful trace elements in some algae species, is found to be dependent

on the growth and method of processing, the *Spirulina* and *Chlorella* (thin cell wall) used for this study were cultivated in indoor photo-bioreactors. The cell wall of *Chlorella* was further ruptured by spray-drying to conserve the nutrients within it and to avoid contaminations. In a similar way, Radhakrishnan *et al.* (2014) reported an increase in the vitamin C and E and a decrease in SOD CAT and LPX which according to the authors, is an indication that the feed was neither toxic nor stressful to postlarvae of *Macrobrachium rosenbergii* Fed dietary *Chlorella* and *Spirulina* at 50% FM level (12.5% dietary inclusion level) Similarly, 5% *Chlorella* powder was found to increase IgM, lysozyme C4 thereby stimulating the immunity of Koi carp (Khani *et al.*, 2017). Thus, to maximise the potentials of algae, the specific attributes of each alga must be considered. For instance, the brominated compounds produced by some red algae like Laurencia and phenolics compound produced by some kelps may render them unsuitable for aquafeed.

5.4 The effects of dietary *Spirulina* and *Chlorella* on growth, fillet composition and antioxidant activities of African catfish (*C. gariepinus*)

Although the antioxidative defence can be reduced by an increase in the level of pollutants, thereby lowering fish production, this problem could be virtually solved by reducing oxidative stress, as well as ensuing damage via dietary supplementation using natural nutraceuticals and antioxidants (Jayakumar *et al.*, 2011). To this effect, this study explored the antioxidant activities and growth performance of African catfish using *S. platensis* and *C. vulgaris* as a substitute for fishmeal being the source of protein in the fish diet. A comparative study was carried out for all the experimental diet. Although the usage of *S. platensis* and *C. vulgaris* as a source of protein for other aquatic animals have been previously investigated (Radhakrishnan *et al.*, 2014; Wu *et al.*, 2016), little has been reported about the relationship between *Spirulina* and antioxidant defence of African catfish. Radhakrishnan *et al.* (2014) previously reported that *S. platensis* and *C. vulgaris*

can be used as a substitute for fishmeal in the diet of *Macrobrachium rosenbergii*. They claimed that the supplemented diet improved the vitamins C and E, and lowered the activities of enzymatic antioxidants (LPx, SOD, and CAT), which indicated that the formulated diets are not toxic and produce no stress to post-larvae.

In this study, an increase in the level of *S. platensis* and *C. vulgaris* supplementation boost the nutrient efficiency and growth of fishes. Although the reduction in feed consumption is not significant, the feed efficiency increases significantly upon supplementation, indicating an encouraging growth response in the juvenile fishes. This could be due to of the high digestibility of *S. platensis* and *C. vulgaris* because of stimulation of the intestinal flora of fish thereby increasing the activity of digestive enzymes resulting in efficient diet utilization (James *et al.*, 2006; Khani *et al.*, 2017). A considerable increase in weight gain was also observed at 75% supplementation for both fresh algae, leading to a higher value of SGR in the fishes Fed with supplemented diets. Furthermore, the observed increase in the value of HSI in fish Fed with the supplemented diets could be because of high lipid and accumulation of glycogen in the liver (Cazenave *et al.*, 2006). This shows the availability of a large amount of food at a favourable aquatic environment for growth for the samples Fed supplemented diet. Fishes with higher HSI values are more energetic because HSI value is related to the performance and size of the liver. The favourability of the environmental condition is also confirmed by the increase in the value of the K factor, which increases significantly upon diet supplementation. The higher growth rate experienced with the fishes Fed supplemented diet is also attributable to the digestibility and the nutritional value of the two microalgae, which were higher when compared with that of the fishmeal.

S. platensis and *C. vulgaris* are a better source of amino acids for African catfish meal than fishmeal (Table 3.9). All the essential amino acids (EAA) are higher in the

supplemented diets except methionine and tryptophan. However, the presence of other amino acids like cysteine is a viable alternative, which could replace about 60 % of methionine needed in the fish diet. Moreover, lysine content decreased upon supplementation with *S. platensis* (Lovell, 1989).

The fish fillet composition followed the same trend as the growth pattern as both algae supplementation resulted in increased fillet protein and lower fat deposition (in contrast with the results obtained with the fingerlings where proximate composition whole body revealed higher flesh fat in SP75%) than the control. There was, however, an inverse relationship between protein and fillet fat deposition, and lipid and moisture as the fillet fat decreased in both algae supplemented diets in a dose-dependent manner with a slight increase in flesh moisture. This result corroborated the findings of Badwy *et al.* (2008) with 50% *Chlorella* diets fed to *Nile tilapia*, 5% (Promya & Chitmanat, 2011) and 15% *Spirulina* supplemented *C. gariepinus,* parrot fish (*Oplegnathus fasciatu*s) respectively (Kim *et al.*, 2013). *Spirulina* has been reported to improve the breakdown of ingested feed materials to extract more nutrients and stimulates the production of enzymes that transport fat for metabolism instead of storing them in the fish body through the stimulation of the intestinal flora of fish (James *et al.*, 2006). Similarly, *Chlorella* powder 2% and 4% were found to have a positive effect on lipid metabolism of juveniles of Olive flounder by reducing the whole-body fat (Kim *et al.*, 2002).

The CAT activities, which correlate with rising H_2O_2 concentration (Wilhelm Filho *et al*., 2005) were found to increase with the increased level of supplementation. Positive relationships were observed between CAT and weight gain of the catfish Fed with diets supplemented with *S. platensis* and *C. vulgaris* (R2 = 0.8107 and 0.8375) respectively, which indicated that improved CAT activities were stimulated by an increase in the growth response because of the high rate of metabolism. This corroborates

with the report of Taufek *et al.* (2016) and Raji *et al.* (2018) which stated that the CAT activity increased in African catfish and, thereby increasing their weight.

The inclusion of *S. platensis* and *C. vulgaris* in the diets had no significant effect on the GST activity but was reduced slightly. This implies that the *Spirulina* and *Chlorella* diets were not toxic to the fish, as they do not contain compounds substances that triggered the stimulation of xenobiotics biotransformation. GST helps to detoxify products of oxidative stress via catalysis of conjugation of a variety of metabolites, which include lipo-peroxidation products and xenobiotic metabolites, with GSH and by converting the toxic complexes into substances that can be discharged more easily.

The activities of SOD slightly increased with an increase in the level of supplementation with SP75 demonstrating the highest activity. This is mainly because the CAT–SOD enzyme mechanism represents the first line of defence against ROS. Therefore, SOD catalyses superoxide anions reduction into H_2O_2, which was subsequently disintegrated by CAT at extra- and intracellular levels (Taufek *et al.*, 2016). The studies of Han *et al.* (2011) and Sahin *et al.* (2014) agree with this trend. SOD is a major antioxidant defence that protects tissues and cells against oxidative stress and ensures immunity against bacterial infection (Xu *et al.*, 2014). The observed increase in the level of SOD indicates that both *S. platensis* and *C. vulgaris* have some bioactive substances capable of regulating the immune response of catfish.

It was revealed that the FA profile in the two freshwater microalgae was distinctively different (Table 3.10). This shows the differences in the quality of food provided by different species of microalga, which is consequential on the consumption responses in the aquatic food web. Microalgae have the highest potential to produce long-chain PUFA when compared with other organisms in the aquatic food web (Krienitz & Wirth, 2006; Norambuena *et al.*, 2015). On the other hand, most animals are unable to

produce essential FAs. The presence of PUFA in the supplemented diet will boost the life cycle of consumer populations, for instance, the development and ontogenetic cycle of catfish. Diet supplementation with *S. platensis* and *C. vulgaris* also boost the EPA (eicosapentaenoic acid, C20:5) content, which according to Tocher (2015), is responsible for the formation of membranes. Apart from being a protein source, *S. platensis* and *C. vulgaris* are also capable of performing a therapeutic function including anti-inflammatory, immunomodulatory, and antioxidant activities, which could play a vital role in animal health. They boost the activity of SOD and CAT, prevent DNA damage and lipid peroxidation, scavenges free radicals, and stimulate cellular antioxidant enzymes (Wu *et al.*, 2016).

CHAPTER 6: CONCLUSION

6.1 Introduction

This thesis investigated the effect of SP and CL as a partial replacement for FM in the diets of African catfish. The digestibility study examined the ADCs of amino acids, fatty acids and macronutrients, for freshwater *Spirulina platensis* (SP) and *Chlorella Vulgaris* (CL) microalgal ingredients in African catfish *(C. gariepinus)* with the use of three diets. These diets included those containing the fish and plant feedstuffs (Reference); and SP or CL (Ref/dried algal powder at 7:3) as test diets. The growth performance study was performed by feeding *C. gariepinus* fingerlings mean weight (7.85 g) with diets formulated with SP or *Chlorella* replacing FM protein at 12.5%, 25%, 50% and 75% graded levels for eight weeks. The fish carcass was also analysed for proximate composition, while fish weight gains were used to compute the optimum replacement levels for best growth. These levels were subsequently used as a guide to formulate feeds for immunity and oxidative stress enzymes studies.

Therefore, based on the findings obtained from these studies, the thesis made the following conclusions:

1. *Spirulina* (SP) and *Chlorella* (CL) meal were well digested by African catfish as they displayed significantly higher ADC for protein, lipid, dry matter, gross energy and essential amino and fatty acids compared to fishmeal used in this study and complied with literature values for plant feedstuffs and fishmeal. However, the quantity and ADCs of histidine, arginine, valine, isoleucine and tryptophan was found to be significantly higher in CL. Methionine, isoleucine, leucine and phenylalanine was also more abundant in CL. It was also found to

be rich in fats, n_3 polyunsaturated fatty acids (PUFA) and docosahexaenoic acid (DHA) contents. It also exhibited significantly highest ADCs for lipid, n_3 and n_6 PUFA and (DHA). On the other hand, SP produced the significantly higher amount of lysine and threonine as well as crude fibre, energy, dry matter and carbohydrate ADCs than CL and FM.

2. Cultivation and feed processing methods (feed extrusion) was found to be responsible for the improved digestibility obtained in the thin cell-walled C. *vulgaris* in this study.
3. *Spirulina* and *Chlorella* could replace up to 75% of fishmeal in African catfish diets due to their improved growth parameters with increasing *Spirulina* and *Chlorella* level. However, the findings of the different experiments conducted in this study showed that *C. gariepinus* fingerlings performed better at 45% whereas the juveniles were better at 35% crude protein level.
4. CL75% dietary replacement level gave the best growth performance, the optimum FM protein replacement with *Spirulina* and *Chlorella* devoid of any negative effect is 68.5% and 69.4% respectively. The effect of *Spirulina* on flesh fat deposition was found to be dependent on the nutritional composition, the lipid source and quantity (plant/ animal oil) as well as the level of *Spirulina* incorporated in the diets.
5. The essential amino acids for every diet were within the range of African catfish requirement except for methionine, phenylalanine and lysine (digestibility study), lysine (growth performance study's SP75%and CL25%; and, phenylalanine in SP75%, CL25% and SP50%). However, cysteine and tyrosine can spare up to 60% and 50% of methionine and phenylalanine respectively. A decrease in crude protein level in (45%) in fingerlings dietary formulations to 35 and 36% for juveniles used for immunity and oxidative stress enzymes studies led to a

decrease in lysine and methionine composition in all the experimental (controls, SP and CL) diets. Suggesting that there is a positive relationship between the CP and amino acids contents of the diets. The protein content of a diet depends on the quantity and quality of its amino acids.

6. Pre-challenge haematological and biochemical indicators also suggested that the values of blood parameters of SP, CL and FM meal Fed fish gave no indications of abnormalities as they were within the normal range of healthy African catfish. Furthermore, since none of the values across the algae experimental diets fell below the normal range peculiar to healthy catfish. Therefore, it is tenable to say that the haematological parameters revealed that catfishes fed with the supplemented diet had better health status than the catfishes fed with fishmeal.
7. The Hgb, Hct, RBC, WBC, total protein albumin and HDL cholesterol levels rose remarkably in SP75% and the groups supplemented with both *Spirulina* and *Chlorella* in a dose-dependent manner in comparison with control. LDL cholesterol significantly reduces with dietary increase in *Spirulina* and *Chlorella* indicating the potentials of both algae to control of atherosclerosis. This is suggesting that the positive effect recorded may be due algae replacement levels than algae type.
8. CL50% gave significantly highest relative per cent survival (RPS) up to 18 days' post-challenge with *Aeromonas hydrophila,* respiratory burst activity (RBA) and numerically highest RBC, Hct, Hgb, platelets, serum protein albumin, globulin and HDL cholesterol when compared to control and other *A. hydrophila* challenged groups. Lysozyme activity (day10 and 18th were, however, higher in the CL75% group.

9. The presence of substantial quantities of EAAs and EFAs especially eicosapentaenoic acid (EPA) docosahexaenoic acid (DHA), and the relatively high post-challenge RBA and Lysozyme activities in *Chlorella* and *Spirulina* meals when compared with the control group, shows that both algae has the potentials to improve the innate immunity of *C.gariepinus* by stimulating the natural protective mechanism that played important roles in reducing mortality caused by *A. hydrophila* injection.
10. Replacement of FM by *Spirulina* and *Chlorella* meal in African catfish diet boosted antioxidant enzyme CAT and SOD (especially in CL75% and SP75% correspondingly) activities of the African catfish despite the minor effect of GST enzymes.
11. Results from this study could provide the basis for the use of *Chlorella* as a replacement for fishmeal protein in *C. gariepinus*. Additionally, the findings could provide additional information to the existing literature regarding the dietary effect of *Spirulina* on this economically important freshwater fish species.

6.2 Future Perspective

The present findings add considerably to increasing knowledge of fish nutritional studies particularly in finding a viable and sustainable alternative to FM as the main protein source in aquafeed. Digestibility is a common problem in algae meals that need to be addressed to create a potential value for algae protein. Nutrient digestibility of *Spirulina* and especially *Chlorella* meal by breaking the rigid cell wall (as was obtained in *C. vulgaris* used for the different studies in this thesis) could be factors that can contribute to increase digestibility and therefore ought to be explored further. Also, the extrusion process of the feed could also be beneficial for increasing digestibility.

Like most plant-based feed, the lysine and methionine contents of most of the diets in this thesis fell below the required amount for African catfish nutrition. However, the inclusion of synthetic lysine and methionine, as well as the high amounts of long-chain PUFA, found in both *Spirulina* and *Chlorella* contributed positively to the growth performance.

Further experimental investigations are needed to estimate other immunological parameters such as phagocytic activity, and bactericidal activity to gain more clarification on non-specific defence mechanism.

The scope of the present study only allowed for investigations of GST, CAT and SOD to determine oxidative stress in *C. gariepinus* fish fed with *Spirulina* and *Chlorella* meal. However, it is known that the oxidation of lipid in the feed could affect the feed palatability. Therefore, additional study of lipid peroxidation and other antioxidative enzymes under various physiological and environmental conditions would be thought-provoking.

A cost-benefit analysis of both algae as compared to FM would be interesting. *Spirulina* and *Chlorella* meal showed potential as fishmeal replacement *C. gariepinus* diets particularly as a sustainable resource because of their well balanced and digestible proteins, amino and fatty acids, and their ability to thrive well in different growth media like open ponds, raceways, wastewater treatment tanks, Sago waste and the likes, thereby reducing the cost of aquaculture production. However, currently, the cost of *Spirulina* and *Chlorella* is the major issue due to the sizes, number and cost of driers to dry the large quantity produced in open raceway ponds. For both microalgae to be seriously considered as routine inputs for aquaculture feeds for catfish, it will require a gigantic scale-up of industrial production to enable consistent products availability and supplied at a price that is competitive with fishmeal and other high protein-base feedstuffs.

Finally, appropriate dietary formulations of *S. platensis* and *C. vulgaris*, as well as sensory quality evaluation of *Spirulina* and *Chlorella* Fed-fish, would improve the marketability of the fish. With more additional research, *Spirulina* and *Chlorella* meal could be a potential primary protein source not only in aqua-feed but also for other livestock and animal diets.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

PUBLICATIONS

Raji, A.A., Alaba, P.A., Yusuf, H., Abu Bakar, N.H., Mohd Taufek, N., Muin, H., Abdul Razak, S. (2018). Fishmeal replacement with *Spirulina platensis* and *Chlorella vulgaris* in African catfish (*Clarias gariepinus*) diet: Effect on antioxidant enzyme activities and haematological parameters. *Research in Veterinary Science, 119*, 67-75.

PRESENTATIONS

Raji, A. A., Oke, M. A., Alias1, Z., Milow1, P., Simarani1, K., & Razak, S. A. (2016). *Effect of Dietary Chlorella And Spirulina On Survival and Haemato-immunological Responses of Clarias gariepinus Juveniles After Aeromonas Hydrophila Challenge.* Paper presented at Universiti Malaysia 13th International Annual Symposium on Sustainability Science and Management (UMTAS2016) Held on 14th- 15th December 2016, Primula Beach Hotel, Kuala Terengganu.

Raji, A. A., Zazali, A., Pozi, M., & Shaharudin, A. R. (2015). *Effect of Partial Replacement of Fishmeal by Spirulina platensis and Chlorella vulgaris on Growth Body Composition and Nutrient Utilization of African Catfish "Clarias gariepinus" (Burchell 1822")*. Paper presented at the 5th International Fisheries Symposium (IFS2015) ASEAN Fisheries 2015: Towards sustainability, advanced technology and community enhancement". organised by Universiti Sains Malaysia (USM) in collaboration with Universiti Malaysia Terengganu (UMT) and Malaysian Fisheries Department. The Gurney Hotel and Residences Penang, Malaysia.