

Thesis for the Degree of Master of Fisheries Science

Effects of three different microalgae on
growth performances and immune responses
in juvenile rainbow trout, *Oncorhynchus*
mykiss

by

Cynthia Lisette Jo Rivero

Division of Fisheries Science

The Graduate School of World Fisheries University,

Pukyong National University

August, 2021

Effects of three different microalgae on growth performances and immune responses in juvenile rainbow trout, *Oncorhynchus mykiss*

세 가지 미세조류가 무지개 송어 (*Oncorhynchus mykiss*)의 성장과 면역반응에 미치는 영향

Advisor: Prof. Sungchul Charles Bai

by

Cynthia Lisette Jo Rivero

A thesis submitted in partial fulfillment of the requirements

For the degree of

Master of Fisheries Science

Division of Fisheries Science, The Graduate School of World Fisheries University
Pukyong National University

August, 2021

Effects of three different microalgae on
growth performances and immune responses
in juvenile rainbow trout, *Oncorhynchus*
mykiss

A thesis

by

Cynthia Lisette Jo Rivero

Approved by:

(Chairperson) Prof. Christopher L. Brown

(Member) Prof. Seunghyung Lee

(Member) Prof. Sungchul C. Bai

August 27, 2021

Effects of three different microalgae on growth performances and immune responses in juvenile rainbow trout, *Oncorhynchus mykiss*

Cynthia Lisette Jo Rivero

Division of Fisheries Science, The Graduate School of World Fisheries University,
Pukyong National University

Abstract

A six weeks feeding trial was conducted to evaluate three different dried microalgae on growth performances and immune responses in juvenile rainbow trout, *Oncorhynchus mykiss*, diets. Twelve groups of fifteen rainbow trout averaging 12.2 ± 0.11 g (mean \pm SD) were randomly distributed into four treatments in triplicates rearing by the semi-recirculation system. Fish were fed four experimental diets such as CONT (a basal diet), CHLO (0.5% *Chlorella sp.*), HAEM (0.5% *Haematococcus sp.*), or SCHIZO (0.5% *Schizochytrium sp.*). Weight gain (%) and specific growth rate of fish fed HAEM diet were significantly higher than those of fed the other diets ($P < 0.05$). Whole-body moisture and crude protein from fish fed CONT and SCHIZO diets were significantly higher than the other diets. Whole-body crude lipid from fish fed HAEM were significantly higher than those from fish fed all the other experimental diets, whole-body ash from fish fed SCHIZO were significantly lower than those from fish fed the other diets ($P < 0.05$). Glucose levels from fish fed CHLO and SCHIZO diets were significantly lower than those from fish fed CONT and HAEM diets. Lysozyme activity from fish fed HAEM diet was significantly higher than those from fish fed the other diets ($P < 0.05$). Fish were challenged by *Vibrio anguillarum* bacteria for 27 days after the feeding trial and the cumulative survival rate (%) from fish fed microalgae (CHLO, HAEM, and SCHIZO) diets were significantly higher than those from fish fed CONT diet ($P < 0.05$). Therefore, these results suggest that microalgae in the diet (CHLO, HAEM, and SCHIZO) could be used as immune enhancers, and dietary HAEM could act as the potential growth promoter in juvenile rainbow trout.

List of tables

Table 1. Proximal composition of the microalgae (% , dry matter basis).....	10
Table 2. Formulation of the basal diet and proximate analysis (% , dry matter basis).....	11
Table 3. Growth performances, feed utilization and survival rates of juvenile rainbow trout fed the experimental diets for 6 weeks	17
Table 4. Whole-body proximate composition of juvenile rainbow trout fed the experimental diets for 6 weeks (% , dry matter basis)	20
Table 5. Biochemical parameters of juvenile rainbow trout fed the experimental diets for 6 weeks.....	21
Table 6. Non-specific immune responses of juvenile rainbow trout fed the experimental diets for 6 weeks.....	23

List of figures

Fig 1. Weight gain of juvenile rainbow trout fed the experimental diets for 6 weeks.....	18
Fig 2. Specific growth rate of juvenile rainbow trout fed the experimental diets for 6 weeks	19
Fig 3. Glucose concentrations in juvenile rainbow trout fed the experimental diets for 6 weeks	22
Fig 4. Lysozyme activity in juvenile rainbow trout fed the experimental diets for 6 weeks.....	24
Fig 5. Cumulative survival rate of rainbow trout juveniles challenged by <i>Vibrio anguillarum</i> (1×10^6 , CFU·mL ⁻¹) for 27 days.....	25

Acknowledgment

I will always be grateful to FAO and the Korean government for allowing me to study in World Fisheries University at Pukyong National University, despite being in a pandemic situation. My deep gratitude and appreciation to my research supervisor, Professor Sungchul C. Bai, for his patience, motivation and guidance through my project and academic life in Korea. I am thankful with Professor Christopher Brown, for his advice, encourage and always positive atmosphere. I also extend my thanks to Professor Seunghyung Lee, for his valuable suggestions and ideas for my dissertation.

This research could not have been done without the support of the Feeds & Foods Nutrition Research Center (FFNREC). Special thanks and appreciation to my lab members, Dr. Ali Hamidohli, Dr. Wonsuk Choi, Dr. Jinho Bae, Hyeong-woo, So-Yeon and Je-in for teaching me lab skills and help me to solve inquiries regarding academic issues. And, to my dear WFU teamwork, Moe, Sam and Zeinab.

I would like to express many thanks to my lovely people (Yura, Milagros, Gloria, Iskander and Sudhanshu) for the wonderful moments in Korea. Forever thankful with Jose Avila, for his appreciated friendship and unconditional support in all aspects. I also thank my best friends in Peru, Kattia, Bárbara and Samuel, for their strong support and positive mood, even though the distance.

I do not even have the words to thank all my family, my driving force, and the main reason to keep going. Endless gratitude to my grandparents, Esperanza Suárez, and Jaime Rivero, for their guidance and care. I cannot thank enough to my mom, Cynthia Rivero, my father Juan Chacaltana, and my little brothers for their support and love. I also extend thanks to my dear uncles Alex, Nancy and Nilton. I dedicated this research to Luis Rivero, Jaime Rivero, who lights my way wherever I go, but most of all, to my new starlight, my wonderful grandmother Esperanza.

Thank you all, truly, from the bottom of my heart.

Table of Contents

Abstract	i
List of tables	ii
List of figures.....	iii
Acknowledgment	iv
Table of Contents.....	v
I. Introduction	1
II. Materials and Methods	5
<i>1. Experimental design and diets</i>	<i>5</i>
<i>2. Experimental fish and feeding trial</i>	<i>5</i>
<i>3. Sample collection and analysis</i>	<i>6</i>
<i>4. Statistical analysis</i>	<i>9</i>
III. Results & Discussion	12
IV. Conclusions and Future directions.....	26
V. References	27
VI. Appendix	35

I. Introduction

Globally, the human population is increasing steadily, and putting pressure on resources, especially in the food sector. Aquaculture is also increasing, becoming the main source of available fish, which currently provides more than 50% of human consumption. Production in 2018 is forecast to increase by 32%, an increase of 26 million tons to 109 million tons by 2030 (FAO, 2020). Aside from having a continuous supply of food, it is also important to identify food sources that are a great nutritional benefit not only for humans but for animals as well (Rasheed et al., 2020). The high nutritional value of the carnivorous freshwater fish, rainbow trout, *Oncorhynchus mykiss*, makes it the second most farmed salmonid species in the world. Global aquaculture production of rainbow trout reached 848 thousand tons in 2018 *FAO (2020)*. However, its intensive expansion became with risks in productivity.

One of the main problems of intensive aquaculture is disease outbreaks (Ma et al., 2020), mostly caused by bacterial infections, such as the *Vibrio* genus, which affects the industry economically (Frans et al., 2011). This leads to the use of high levels of antibiotics, resulting in negative environmental impacts. (Han et al., 2019), antibiotic-resistant bacteria (Dawood et al., 2018) and antibiotic residues in animals and humans (Yuan et al., 2019), allowing some countries to prohibit or restrict the use of antibiotics in feeds used as growth promoters (Gamboa-Delgado

& Márquez-Reyes, 2018; Zhou et al., 2021) and/or immune enhancers (Lulijwa et al., 2020). For these reasons, increased emphasis is being placed on the concepts of antibiotic-free feeds, organic farming, and food safety.

The world is changing, especially in food consumers, giving more attention to the environment, animal ethics, and animal welfare. From the production point of view, the industry must increase its volume but at the same time decrease its cost and environmental impact. Thus, researchers and farmers are focusing on the use of natural additives to reduce the use of antibiotics and metals in aquaculture and improve the growth and stimulate the immune response of fish as well without affecting productivity (Bai et al., 2015; Cho & Lee, 2012; Zhao et al., 2020). It is worth noting, the increase in demand for organic aquaculture is leading to the consideration of organic trout production (Ortiz-Chura et al., 2018) and microalgae additive is a suitable source for this purpose (M. R. Shah et al., 2018; Yarnold et al., 2019).

Additives such as prebiotics, probiotics and immunostimulants have been evaluated in fish nutrition. Currently, studies with immunostimulants are increasing, and yeast, plants, bacteria, algae, and microalgae can be used (Thépot et al., 2020). One of the most important applications of microalgae in aquaculture is related to nutrition (Christaki et al., 2017; Muller-Feuga, 2000) as live feeds (Sirakov et al., 2015) or powder addition, as it can promote growth, survival, immune system, and improve the nutritional value and quality of fish products

(Milan et al., 2021) due to this unicellular organism provides nutrients such as proteins, polysaccharides and polyunsaturated fatty acid (Christaki et al., 2011), pigments (Becker, 2013; J. Li et al., 2015), antioxidants (Taghavi Takyar et al., 2019) and other bioactive compounds (Zhang et al., 2020).

Kaparapu (2018) mentioned the some of the most frequent species used in formulated feed ingredients are *Chlorella vulgaris* and *Haematococcus pluvialis*. *Chlorella sp.* is a freshwater algae which contains protein (51%-58%), and the remained consists of carbohydrate, lipid and other components (Panahi et al., 2019), which facilitates easy digestion (Rani et al., 2018). *Haematococcus sp.* is green algae with high levels of astaxanthin mainly used as a dietary supplement for pigmentation of salmonids for many years (Choubert et al., 2009; H. Li & Liu, 2019) and recently has more attention as antioxidant due to its biological properties (Lorenz & Cysewski, 2000; Mularczyk et al., 2020). In addition, Lim et al., (2018) mentioned that this additive also increase reproductive capacity, stress tolerance, survival, disease resistance, and growth performances. *Schizochytrium sp.* is a heterotrophic algae that is an emerging source of polyunsaturated fatty acids (n-3 LC-PUFA) in aquafeeds because it is rich in DHA, for this reason, it has been tested to replace fish oil (Allen et al., 2019; Bélanger-Lamonde et al., 2018) and fishmeal, however, Tibbetts et al., (2020) mentioned that the dietary inclusion of *Schizochytrium sp.* meal is limited (<10%), as previous studies showed that growth performance and digestibility were affected in fish. Hence, trials using this

microalga as a dietary supplement in aquaculture are scarce, especially in terms of immune response and histological parameters (de Souza et al., 2020).

Due to the above-mentioned benefits, studies have been conducted with microalgae as feed additive showing significant improvement in growth, feed utilization, immune response, and disease resistance in aquatic animals using *Chlorella sp.* (Kim et al., 2002; Mahmoud et al., 2020; Xu et al., 2014), *Haematococcus sp.* (Ju et al., 2012; Sheikhzadeh et al., 2012; J. Xie et al., 2020; S. Xie et al., 2018; Yu et al., 2020) and *Schizochytrium sp.* (Lyons et al., 2017; Sarker et al., 2020; M. R. Shah et al., 2018; J. Xie et al., 2019). According to Skalli et al., (2020), the use of microalgae as a supplement in diets is becoming promising for high-value fish, however, studies in salmonids species is still limited, especially in rainbow trout.

Therefore, this study was done to evaluate the effects of dietary supplementation of three different microalgae (*Chlorella sp.*, *Haematococcus sp.* and *Schizochytrium sp.*) as a natural feed additive in juvenile rainbow trout diets on growth performances, serum biochemical parameters, immune system, and disease resistance to the bacterial infection of *Vibrio anguillarum*.

II. Materials and Methods

1. Experimental design and diets

Four diets were formulated to be iso-nitrogenous (51% crude protein) and iso-lipidic (13% lipid). A basal diet (CONT) and other three diets with the supplementation of 0.5% of microalgae, such as *Chlorella sp.* (CHLO), *Haematococcus sp.* (HAEM) and *Schizochytrium sp.* (SCHIZO). The proximate composition of CONT diet is presented in Table 1. Formulated diets were based on sardine fish meal, soybean meal, blood meal, and wheat gluten meal as the main protein sources; fish oil as the lipid source; meat and bone and wheat flour as the carbohydrate sources. Microalgae meals were obtained from FIRA, Korea Fisheries Resources Agency, Busan, South Korea. Diets were prepared following the procedure of Bai & Kim (1997), starting with the weighing, and mixing of all the dry ingredients using the HYVM-1214, Hanyoung Food Machin, fish oil, and 30% water were added, then the feed was pelletized (2 mm) using the SMC-12, SUN Engineering and dried at room temperature for 48 hours. After processing, the feed was stored at -20°C in a freezer until use.

2. Experimental fish and feeding trial

Juvenile rainbow trout were derived from Hyangchon, Chungju - Chungcheong, South Korea fish farm to Feeds and Foods Nutrition Research Center (FFNRC) at Pukyong National University. Acclimatization was for 1 week feeding with basal

diet, after that, one hundred eighty fish with initial body weight 12.16 ± 0.01 g were randomly allocated into 12 tanks of 30 L. Each tank contained 15 fish with three replicates by an indoor semi-recirculating system receiving a constant water flow ($2 \text{ L}\cdot\text{min}^{-1}$). Seventy percent of the water was replaced every day with water suitable for fish. The water temperature was monitored 4 times per day and maintained at 15.5 ± 1 °C, the dissolved oxygen was near saturation using air stone in each tank under a photoperiod of 12L: 12 D. Fish feeding frequency was three times a day (09:00, 12:00 and 17:00) with a rate of 3% of the stocked biomass for 6 weeks. In addition, to remove uneaten feed and feces, all aquaria were siphoned daily.

3. Sample collection and analysis

Growth performances

The total number of fish and weight were evaluated at the beginning and at the end of the trial. After the experiment, all fish fasted for 24 hours. A random selection of three fish per tank was made for biometry and anesthetized with, 100 ppm of Tricaine methanesulphonate, Sigma, (MS-222), and the data obtained were used to calculate growth performance and feed utilization, after that, sampling was dissected to obtain liver and viscera for analysis of hepatosomatic index and visceromatic index, respectively.

All parameters were calculated following the equations below:

- ✓ Weight gain (WG, %) = (final wt. - initial wt.) / initial wt × 100
- ✓ Specific growth rate (SGR, %) = (ln final wt. - ln initial wt.) × 100 / days
- ✓ Feed efficiency ratio (FE, %) = (wet weight gain / dry feed intake) × 100
- ✓ Protein efficiency ratio (PER) = (wet weight gain / protein intake)
- ✓ Condition factor = (wet weight / total length³) × 100
- ✓ Hepatosomatic index (HSI, %) = liver wt. / body wt. × 100
- ✓ Viscerosomatic index (VSI, %) = viscera wt. / body wt. × 100

Proximate analysis

Proximal composition analyses of diets and fish whole-body were determined according to the standard methods of AOAC (2005). Moisture content from feed and fish were estimated by samples dried at 105 °C for 24h to a constant weight. Ash was performed by incineration using a muffle at 550 °C for 3h. To quantify protein and lipid content, samples were frozen at -20 °C for 24h, and then freeze-drying for 20h using a machine (Advantage 2.0, VirTis, New York, USA). Crude protein was measured following the Kjeldahl method (N × 6.25) using the Auto Kjendahl system (Buchi B-324/435/412, Switzerland; Metrohm 8-719/806, Switzerland) and crude lipid was analyzed by Soxhlet extraction with the Soxhlet system 1046 (Tacator AB, Hoganas, Sweden). All chemical analyses were carried out using a sample of three fish per tank and feed.

Serum biochemical parameters

With the aim of evaluating the impact of diets on hematological parameters, five fish per tank were used to collect blood from the caudal vein using a 1 mL non-heparinized syringe. Blood samples were centrifuged at 5000 x g for 10 minutes to separate the serum, after that, samples were stored at -70 °C for the determination of aspartate transaminase (AST), alanine transaminase (ALT), glucose (GL), and total protein (TP) using Fuji DRI-CHEM 3500i (Fuji Photo Film LTd, Tokyo, Japan) machine.

Non-specific immune responses

The second set of serum was used for the analysis of Lysozyme activity and Myeloperoxidase (MPO) activity. The lysozyme activity of the serum was analyzed by the turbidimetric assay following the procedure of HULTMARK et al., (1980) with some modification. 0.1 ml of serum was added to 2 ml of *Micrococcus lysodeikticus* (0.2 mg/ml) in a 0.05 M of sodium phosphate buffer at pH 5.52. The mixed suspension was added in 96 well plate and the absorbance at 450 nm of wavelength was assessed between 0 and 30 minutes using a microplate reader. Lysozyme activity levels were determined as the amount of serum causing a reduction in absorbance of 0.001 min⁻¹. Total myeloperoxidase (MPO) activity was determined according to the method of Quade and Roth (1997). Briefly, 20 µl of serum was diluted in 80 µl Hanks Balanced Salt Solution (HBSS) without Ca²⁺ or Mg²⁺ into a 96-well plate and then 35 µL of 3, 3', 5, 5' tetramethylbenzidine

hydrochloride (TMB, 20 mM; Sigma-Aldrich) and H₂O₂ (5 mM) were subsequently added into that plate. This plate was incubated at 30°C for 2 min and 35 µl 4M H₂SO₄ was added to stop the color-reaction and the absorbance was read at 450 nm using a microplate reader.

Challenge test

At the end of the experimental feeding trial, five fish per tank were randomly collected and injected via intraperitoneal with 0.1 ml of bacterial pathogen suspension (*Vibrio anguillarum*) at a concentration of 1×10^6 colony forming units (CFU·mL⁻¹), and then distributed into 12 tanks with a capacity of 30 L each one. The water condition was maintained at a temperature of 15.5 ± 1 °C (mean \pm SD) and constant oxygen. Mortality was recorded every day for 27 days to calculate survival. Fish were not fed during the period of the challenge test.

4. Statistical analysis

Data were processed and analyzed by one-way ANOVA using the IBM SPSS 26 statistical program, after normality and homogeneity of variance tests, with the purpose of statistically comparing the effects of the experimental diets. The Least Significant Difference (LSD) test was used when significant differences were obtained ($p < 0.05$).

Table 1. Proximal composition of the microalgae (% , dry matter basis)¹

Ingredients	<i>Chlorella</i> <i>sp.</i>	<i>Haematococcus</i> <i>sp.</i>	<i>Schizochytrium</i> <i>sp.</i>
Moisture (%)	3.83 ± 0.11	3.11 ± 0.16	3.50 ± 0.06
Crude protein (%)	58.5 ± 0.32	15.9 ± 0.18	25.5 ± 0.17
Crude lipid (%)	1.05 ± 0.05	26.8 ± 0.00	50.5 ± 0.43
Crude ash (%)	4.07 ± 0.03	8.24 ± 0.03	15.4 ± 0.19

¹ Values are mean (± SD) of duplicate samples.

Table 2. Formulation of the basal diet and proximate analysis (% , dry matter basis)

Ingredients	%
Sardine Fish meal ¹	22.0
Soybean meal ¹	17.5
Blood meal ¹	4.70
Wheat gluten meal ¹	5.00
Poultry byproduct ¹	16.2
Wheat flour ¹	13.9
Meat and bone meal ¹	9.20
Fish oil ¹	6.80
Mineral Mix ²	2.00
Vitamin Mix ³	2.00
Choline ¹	0.04
Lecithin ¹	0.20
Cellulose ¹	0.50
Total	100,0
<i>Proximate analysis (% of DM basis)</i>	
Moisture	5.52
Crude protein	51.3
Crude lipid	13.2
Crude ash	12.2

¹ Sigma-Aldrich Korea Yongin, Republic of Korea; Cellulose was replaced by the graded level of microalgae (*Chlorella sp.*, *Haematococcus sp.* and *Schizochytrium sp.*)

² Contains (as mg.kg⁻¹ in diets): Ascorbic acid, 300; dl-Calcium pantothenate, 150; Choline bitartrate, 3000; Inositol, 150; Menadione, 6; Niacin, 150; Pyridoxine HCl, 15; Riboflavin, 30; Thiamine mononitrate, 15; dl-a-tocopherol acetate, 201; Retinyl acetate, 6; Biotin, 1.5; Folic acid, 5.4; Cobalamin, 0.06.

³ Contains (as mg.kg⁻¹ in diets): NaCl, 437.4; MgSO₄·7H₂O, 1379.8; ZnSO₄·7H₂O, 226.4; Fe-Citrate, 299; MnSO₄, 0.016; FeSO₄, 0.0378; CuSO₄, 0.00033; Calcium iodate, 0.0006; MgO, 0.00135; NaSeO₃, 0.00025.

III. Results & Discussion

Growth performances

Six weeks after feeding trial and statistical analysis from the data obtained, figures 1 and 2 shown that weight gain (WG, %) and specific growth rate (SGR $\% \cdot \text{day}^{-1}$) of juvenile rainbow trout fed HAEM diet were significantly higher than those fish fed with the experimental diets ($P < 0.05$). Similar findings were observed in previous studies in carnivorous fish such as yellow croacker, *Pseudosciaena crocea*, at levels of 0.28, 0.56 and 1.12 g 100 g⁻¹ diet (M. Li et al., 2014); golden pompano, *Trachinotus ovatus*, at the level of 0.3% (J. Xie et al., 2020; S. Xie et al., 2018) and spotted sea bass, *Lateolabrax maculatus*, in diets containing levels of inclusion from 0.4 to 1.0% (Yu et al., 2020). The improvement on WG and SGR in fish fed with *Haematococcus sp.* may be stimulated due to its favorable nutritional composition or its ability to accumulate up to 5% DW of astaxanthin, which is beneficial for the fish metabolism and digestibility (Ju et al., 2012; M. M. R. Shah et al., 2016). In this study, WG and SGR of fish fed CHLO and SCHIZO diets were not significantly different from those of fed HAEM and CONT diets. Lyons et al., (2017) reported similar results when fish were fed with 5% of *S. limacinum*. By contrast, Kim et al., (2002) observed that WG, and SGR were significantly higher in juvenile japanese flounder, *Paralichthys olivaceus*, fed at 2% inclusion of *C. ellipsoidea* meal in their diet. Also, WG and SGR were significantly higher in

tilapia feed at 10% of *C. vulgaris* (Galal et al., 2018). However, a significant decrease in SGR was found using *C. vulgaris* in the tilapia diet compare with those fish fed CONT diet (Hossain et al., 2017).

Results of feed efficiency (FE,%), protein energy ratio (PER), and condition factor (CF) in all experimental diets are shown in Table 3. No significant differences were found among the treatments. Our findings agree with previous research with fish fed microalgae (M. Li et al., 2014; J. Xie et al., 2020).

The hepatosomatic index (HIS,%), viscerosomatic index (VSI,%), and survival rate (%) were not significant different in all experimental diets (Table 3). Fadl et al., (2017) also reported no significant differences in those parameters in tilapia fed with microalgae.

Proximate analysis

Significant differences ($P>0.05$) were found in the whole-body proximal composition of fish fed all experimental diets (Table 4). Whole-body moisture and crude protein from fish fed CONT & CHLO diets were significantly higher than those from fish fed HAEM & SCHIZO diets. Whole-body crude lipid from fish fed HAEM were significantly higher than those of fish fed all other diets. Similar results were found in shrimp fed diets with Haematococcus inclusion of 0.28, 0.26 and 1.12% in the diets (Li et al., 2014). Whole-body lipid from fish fed SCHIZO diets were significantly higher than those from fish fed CHLO &

CONT diets ($P < 0.05$). Crude ash in body composition from fish fed SCHIZO were significantly lower than all experimental diets ($P < 0.05$). These results are in accordance with Allen et al., (2019), where shrimp tended to have a higher lipid content in the diets using Schizochytrium, in addition, they had a significantly lower moisture and ash content when compared to the control diet.

Serum biochemical parameters

Parameters such as aspartate transaminase (AST), alanine transaminase (ALT) and total protein (TP) and glucose (GLU) are shown in table 5. Serum biochemical parameters are related to fish health and their immune response. Activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are enzymes that measure changes in the liver and indicators of tissue damage. Those enzymes were not affected significantly in fish fed all treatments, indicating that the liver function of the fish was not impaired. The results of ALT and AST in this study are in accordance with Fadl et al., (2017) and Mahmoud et al., (2020) when tilapia were fed with *C. Vulgaris*. Glucose (GLU) and total protein (TP) were chosen to understand the health status and metabolism of fish. Figure 3 shown that serum glucose levels of fish fed CHLO and SCHIZO diets were significantly lower compared to fish fed CONT and HAEM diets ($P < 0.05$). The TP concentrations measured in all diets were found to be within normal physiological ranges.

Non-specific immune responses

Xu et al. (2010) mention that non-specific immune parameters are indicators of health, as they maintain the balance between the release and elimination of reactive oxygen species in immune cells. MPO and lysozyme play an important role in their bactericidal activity and disease resistance (Magnadóttir, 2006). The myeloperoxidase (MPO) results produced by phagocytes of fish fed HAEM and SCHIZO diets were slightly higher than those fish fed CONT diet, however, statistically, no significant differences between all experimental diets were found (Table 6).

Lysozyme activity levels of fish fed with the different diets are shown in figure 4. HAEM treatment indicates a significantly elevated lysozyme activity, a fact that reflects an important humoral indicator of non-specific immunity in the fish and a better antioxidant function. However, the addition of *Chlorella sp.* and *Schizochytrium sp.* in rainbow trout diets did not show significant differences in lysozyme activity; similar results were found in fish fed with 10% of *Chlorella vulgaris* (Galal et al., 2018; Saberi et al., 2017). In contrast, lysozyme levels increased significantly when 0.4 to 2.0% of *Chlorella. vulgaris* were added in diets of gibel carp, *Carassius auratus gibelio* (Xu et al., 2014). Also, the supplementation of 5 % of *Chorella vulgaris* increased lysozyme levels and stimulate fish immune status in tilapia (Mahmoud et al., 2020) and koi carp, *Cyprinus carpio* (Khani et al., 2017). Considering the previous studies, it could

be inferred that to notice a significant difference in the non-specific parameters, the *Chlorella sp.* and *Schizochytrium sp.* dietary inclusion should be increased.

Challenge test

After 6 weeks of the feeding trial, juvenile rainbow trout were challenged with *Vibrium anguillarum* for 27 days. Figure 5 presents the cumulative survival rate of each diet and the days after injection, where starting at day 24 it is observed that rainbow trout fed with three different microalgae were significantly higher against *V. anguillarum* than those fish fed a basal diet. The cumulative survival rate of fish fed the HAEM diet achieved 80%, resulting in the best results at the end of the challenge test when compared with all experimental diets, where CHLO and SCHIZO reached 60% and CONT diet 20%. An advantage of HAEM diet might be due to the *Haematococcus* compound, such as the red-orange carotenoid astaxanthin, which functions as an anti-inflammatory and antioxidant, increasing the survival of the fish (Vallejos-Vidal et al., 2016).

Table 3. Growth performances, feed utilization and survival rates of juvenile rainbow trout fed the experimental diets for 6 weeks¹

	Diets ²				Pooled SEM ¹¹
	CONT	CHLO	HAEM	SCHIZO	
WG ³	83.1 ^b	78.3 ^b	99.4 ^a	71.3 ^b	5.98
SGR ⁴	1.68 ^b	1.61 ^b	1.92 ^a	1.49 ^b	0.09
FE ⁵	73.3 ^{ns}	68.4	72.8	65.3	1.91
PER ⁶	1.43 ^{ns}	1.32	1.46	1.30	0.04
HSI ⁷	1.14 ^{ns}	1.20	1.10	1.17	0.02
VSI ⁸	4.74 ^{ns}	4.74	4.78	4.81	0.02
CF ⁹	1.02 ^{ns}	1.01	1.01	0.99	0.03
Survival ¹⁰	93.3 ^{ns}	86.7	84.4	95.6	2.65

¹ Values are means from triplicate groups of rainbow trout where the values in each row with different superscripts are significantly different ($P < 0.05$).

² Diets represent CONT = Control, CLHO = *Chlorella sp.*, HAEM = *Haematococcus sp.*, SCHIZO = *Schizochytrium sp.*

³ Weight gain (WG, %) = (final wt. - initial wt.) × 100 / initial wt

⁴ Specific growth rate (SGR, %·day⁻¹) = (ln final wt. - ln initial wt.) × 100 / days

⁵ Feed efficiency ratio (FE, %) = (wet weight gain / dry feed intake) × 100

⁶ Protein efficiency ratio (PER) = (wet weight gain / protein intake)

⁷ Hepatosomatic index (HSI, %) = liver wt. × 100 / body wt.

⁸ Viscerosomatic index (VSI, %) = viscera wt. × 100 / body wt.

⁹ Condition factor = (wet weight / total length³) × 100

¹⁰ Survival (%) = (total fish - dead fish) × 100 / total fish

¹¹ Pooled standard error of mean: $SD \cdot \sqrt{n}^{-1}$

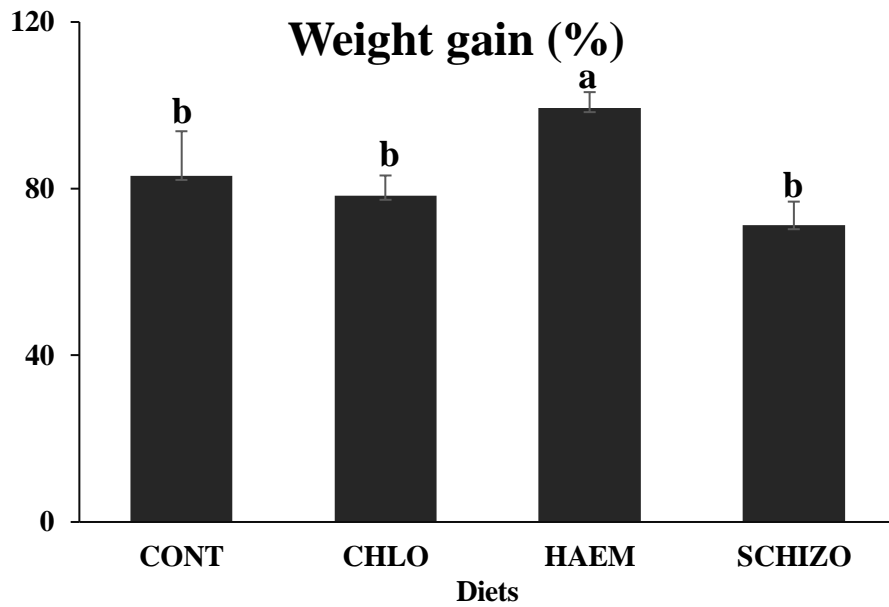


Fig 1. Weight gain of juvenile rainbow trout fed the experimental diets for 6 weeks. Diets represent CONT = Control, CLHO = *Chlorella sp.*, HAEM = *Haematococcus sp.*, SCHIZO = *Schizochytrium sp.*

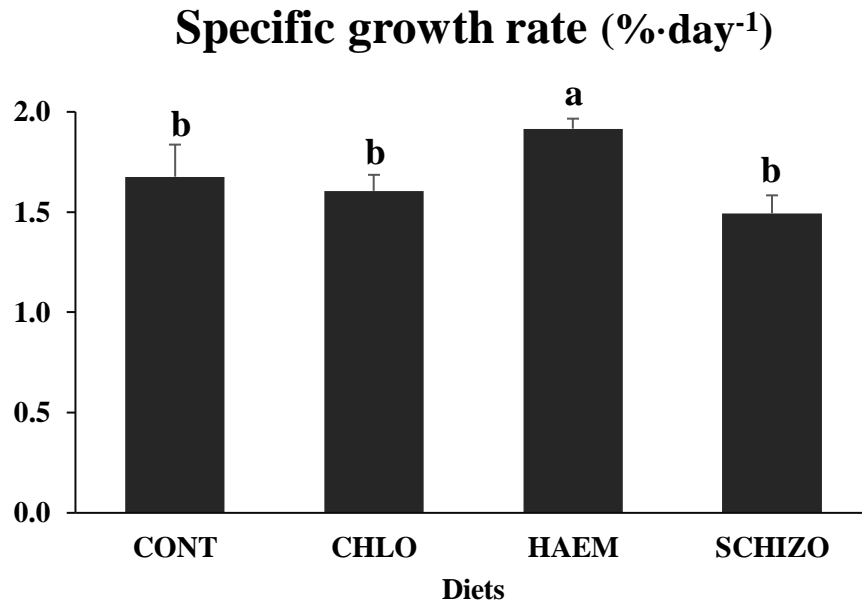


Fig 2. Specific growth rate of juvenile rainbow trout fed the experimental diets for 6 weeks. Diets represent CONT = Control, CLHO = *Chlorella sp.*, HAEM = *Haematococcus sp.*, SCHIZO = *Schizochytrium sp.*

Table 4. Whole-body proximate composition of juvenile rainbow trout fed the experimental diets for 6 weeks (% , dry matter)¹

	² Diets				Pooled SEM
	CONT	CHLO	HAEM	SCHIZO	
Moisture (%)	76.7 ^a	76.1 ^a	75.4 ^b	75.5 ^b	0.19
Crude Protein (%)	71.7 ^a	71.4 ^a	68.2 ^b	68.3 ^b	0.60
Crude Lipid (%)	17.2 ^c	17.4 ^c	21.8 ^a	20.3 ^b	0.70
Crude Ash (%)	11.3 ^a	11.5 ^a	10.8 ^a	9.99 ^b	0.21

¹Values are means from triplicate groups of rainbow trout where the values in each row with different superscripts are significantly different (P<0.05).

² Diets represent CONT = Control, CLHO = *Chlorella sp.*, HAEM = *Haematococcus sp.*, SCHIZO = *Schizochytrium sp.*

Table 5. Biochemical parameters of juvenile rainbow trout fed the experimental diets for 6 weeks¹

	Diets ²				Pooled
	CONT	CHLO	HAEM	SCHIZO	SEM ⁷
AST ³	38.4 ^{ns}	43.5	45.6	52.9	30.1
ALT ⁴	8.67 ^{ns}	8.67	6.33	8.00	0.55
GL ⁵	82.3 ^a	73.7 ^b	83.3 ^a	70.3 ^b	3.21
TP ⁶	3.47 ^{ns}	3.70	3.70	5.40	0.08

¹ Values are means from triplicate groups of fish where the values in each row with different superscripts are significantly different ($P < 0.05$)

² Diets represent CONT = Control, CLHO = *Chlorella sp.*, HAEM = *Haematococcus sp.*, SCHIZO = *Schizochytrium sp.*

³ AST (U·L⁻¹) = Aspartate transaminase

⁴ ALT (U·L⁻¹) = Alanine transaminase

⁵ GL (mg·dL⁻¹) = Glucose

⁶ TP (g·dL⁻¹) = Total Protein

⁷ Pooled standard error of mean: $SD \cdot \sqrt{n}^{-1}$

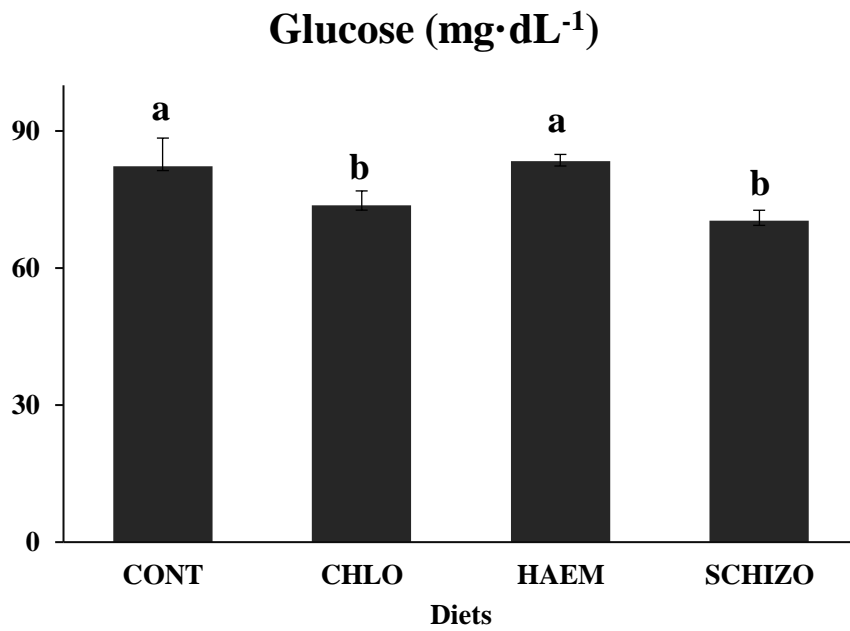


Fig 3. Glucose concentrations in juvenile rainbow trout fed the experimental diets for 6 weeks. Diets represent CONT = Control, CLHO = *Chlorella sp.*, HAEM = *Haematococcus sp.*, SCHIZO = *Schizochytrium sp.*

Table 6. Non-specific immune responses of juvenile rainbow trout fed the experimental diets for 6 weeks¹

	Diets ²				Pooled SEM ⁵
	CONT	CHLO	HAEM	SCHIZO	
MPO ³	1.17 ^{ns}	1.18	1.29	1.23	0.03
LYZ ⁴	2.09 ^b	1.88 ^b	5.08 ^a	1.99 ^b	0.78

¹ Values are means from triplicate groups of fish where the values in each row with different superscript are significantly different ($P < 0.05$).

² Diets represent CONT = Control, CLHO = *Chlorella sp.*, HAEM = *Haematococcus sp.*, SCHIZO = *Schizochytrium sp.*

³ MPO (% inhibition) = Superoxide dismutase activity

⁴ Lysozyme (U·mL⁻¹) = Lysozyme activity

⁵ Pooled standard error of mean: $SD \cdot \sqrt{n}^{-1}$

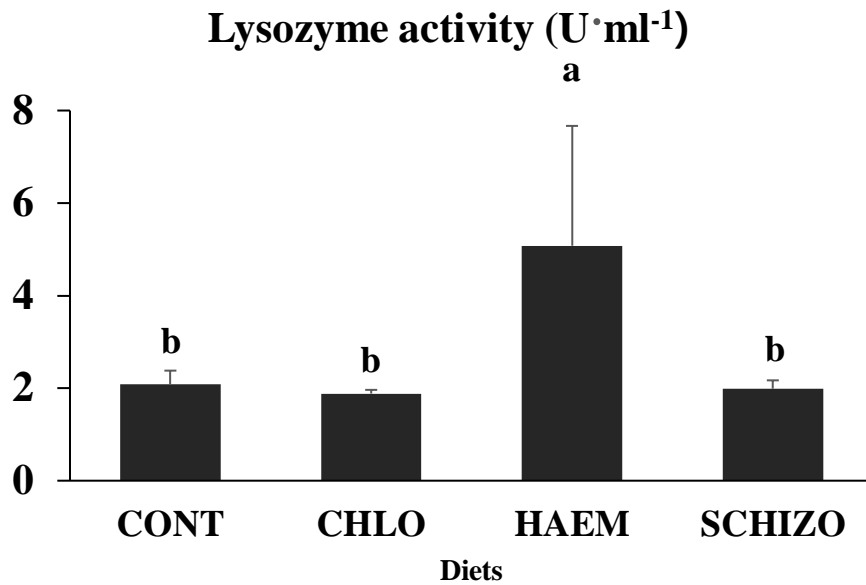


Fig 4. Lysozyme activity in juvenile rainbow trout fed experimental diets for 6 weeks. Diets represent CONT = Control, CLHO = *Chlorella sp.*, HAEM = *Haematococcus sp.*, SCHIZO = *Schizochytrium sp.*

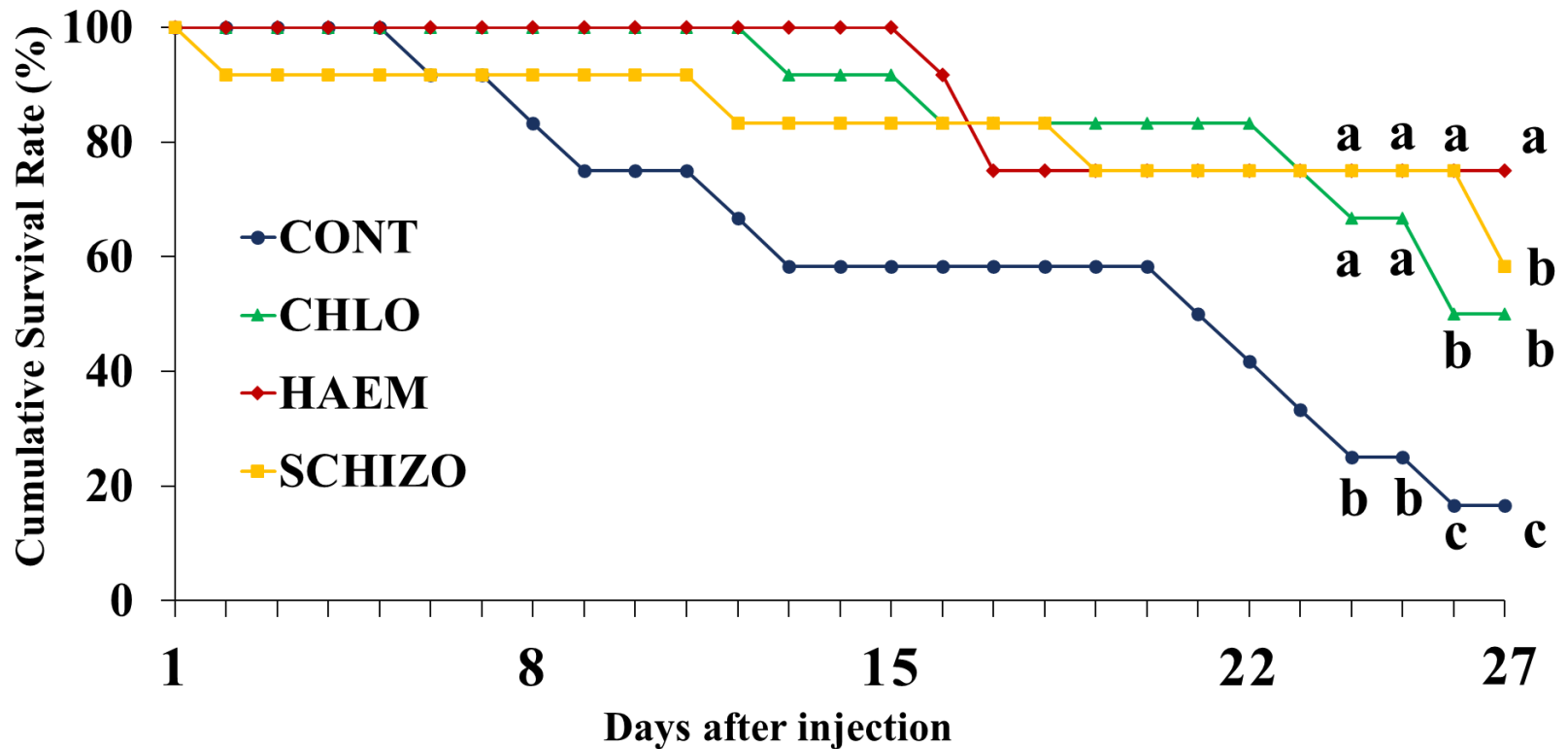


Fig 5. Cumulative survival rates of rainbow trout juveniles challenged by *Vibrio anguillarum* (1×10^6 , CFU·mL⁻¹) for 27 days.

Diets represent CONT = Control, CLHO = *Chlorella sp.*, HAEM = *Haematococcus sp.*, SCHIZO = *Schizochytrium sp.*

IV. Conclusions & Future research

The inclusion of microalgae in the diets tested in this study (0.5% *Chlorella sp.*; *Schizochytrium sp.* or *Haematococcus sp.*) could be used as a feed additive to improve the immune system of the fish. Moreover, the inclusion of 0.5% *Haematococcus sp.* could also be considered the growth promoter in juvenile rainbow trout.

Future Studies using *Haematococcus sp.* as a feed additive:

First, determine optimal dietary levels of *Haematococcus sp.* in juvenile rainbow trout and evaluate growth performances and non-specific immune responses. Then, once the appropriate dose has been found, evaluate the effects of the *Haematococcus sp.* diet on the immune system, e.g., by gut histology and gut microbiota since in fish, the gut is a key element in food digestion and nutrient absorption. Also analyze the pancreatic digestive enzymes of fish, as they are considered indicators of the digestive capacity of fish. Finally, to evaluate changes in the quality of fish fillets, parameters such as flesh color and flavor. Since carotenoids are widely used as a food additive in the salmonid sector, mainly as a pigment to ensure the quality characteristics of the meat obtained, *Haematococcus sp.* contains high levels of astaxanthin and lipids.

V. References

- Allen, K. M., Habte-Tsion, H. M., Thompson, K. R., Filer, K., Tidwell, J. H., & Kumar, V. (2019). Freshwater microalgae (*Schizochytrium sp.*) as a substitute to fish oil for shrimp feed. *Scientific Reports*, 9(1). <https://doi.org/10.1038/s41598-019-41020-8>
- Bai, S. C., Katya, K., & Yun, H. (2015). 7 – Additives in aquafeed: An overview. In *Feed and Feeding Practices in Aquaculture*.
- Becker, E. W. (2013). Microalgae for Aquaculture: Nutritional Aspects. In *Handbook of Microalgal Culture: Applied Phycology and Biotechnology: Second Edition*. <https://doi.org/10.1002/9781118567166.ch36>
- Bélanger-Lamonde, A., Sarker, P. K., Ayotte, P., Bailey, J. L., Bureau, D. P., Chouinard, P. Y., Dewailly, É., Leblanc, A., Weber, J. P., & Vandenberg, G. W. (2018). Algal and Vegetable Oils as Sustainable Fish Oil Substitutes in Rainbow Trout Diets: An Approach to Reduce Contaminant Exposure. *Journal of Food Quality*, 2018. <https://doi.org/10.1155/2018/7949782>
- Cho, S. H., & Lee, S. M. (2012). Onion Powder in the Diet of the Olive Flounder, *Paralichthys olivaceus*: Effects on the Growth, Body Composition, and Lysozyme Activity. *Journal of the World Aquaculture Society*, 43(1). <https://doi.org/10.1111/j.1749-7345.2011.00489.x>
- Choubert, G., Cravedi, J. P., & Laurentie, M. (2009). Effect of alternate distribution of astaxanthin on rainbow trout (*Oncorhynchus mykiss*) muscle pigmentation. *Aquaculture*, 286(1–2), 100–104. <https://doi.org/10.1016/j.aquaculture.2008.09.001>
- Christaki, E., Florou-Paneri, P., & Bonos, E. (2011). Microalgae: A novel ingredient in nutrition. *International Journal of Food Sciences and Nutrition*, 62(8). <https://doi.org/10.3109/09637486.2011.582460>
- Christaki, E., Karatzia M, & Florou-Paneri, P. (2017). The use of algae in animal nutrition. *Journal of the Hellenic Veterinary Medical Society*, 61(3). <https://doi.org/10.12681/jhvms.14894>
- Dawood, M. A. O., Koshio, S., & Esteban, M. Á. (2018). Beneficial roles of feed

- additives as immunostimulants in aquaculture: a review. In *Reviews in Aquaculture* (Vol. 10, Issue 4). <https://doi.org/10.1111/raq.12209>
- de Souza, F. P., de Lima, E. C. S., Urrea-Rojas, A. M., Suphoronski, S. A., Facimoto, C. T., da Silva Bezerra, J., de Oliveira, T. E. S., de Pádua Pereira, U., Di Santis, G. W., de Oliveira, C. A. L., & Lopera-Barrero, N. M. (2020). Effects of dietary supplementation with a microalga (*Schizochytrium sp.*) on the hemato-immunological, and intestinal histological parameters and gut microbiota of Nile tilapia in net cages. *PLoS ONE*, *15*(1). <https://doi.org/10.1371/journal.pone.0226977>
- Fadl, S. E., ElGohary, M. S., Elsadany, A. Y., Gad, D. M., Hanaa, F. F., & El-Habashi, N. M. (2017). Contribution of microalgae-enriched fodder for the Nile tilapia to growth and resistance to infection with *Aeromonas hydrophila*. *Algal Research*, *27*. <https://doi.org/10.1016/j.algal.2017.08.022>
- FAO. (2020). The State of World Fisheries and Aquaculture 2020. In brief. *The State of World Fisheries and Aquaculture 2020. In Brief*. <https://doi.org/10.4060/ca9231en>
- Frans, I., Michiels, C. W., Bossier, P., Willems, K. A., Lievens, B., & Rediers, H. (2011). *Vibrio anguillarum* as a fish pathogen: Virulence factors, diagnosis and prevention. In *Journal of Fish Diseases* (Vol. 34, Issue 9). <https://doi.org/10.1111/j.1365-2761.2011.01279.x>
- Galal, A. A. A., Reda, R. M., & Abdel-Rahman Mohamed, A. (2018). Influences of *Chlorella vulgaris* dietary supplementation on growth performance, hematology, immune response and disease resistance in *Oreochromis niloticus* exposed to sub-lethal concentrations of penoxsulam herbicide. *Fish and Shellfish Immunology*, *77*. <https://doi.org/10.1016/j.fsi.2018.04.011>
- Gamboa-Delgado, J., & Márquez-Reyes, J. M. (2018). Potential of microbial-derived nutrients for aquaculture development. In *Reviews in Aquaculture* (Vol. 10, Issue 1). <https://doi.org/10.1111/raq.12157>
- Han, P., Lu, Q., Fan, L., & Zhou, W. (2019). A review on the use of microalgae for sustainable aquaculture. *Applied Sciences (Switzerland)*, *9*(11). <https://doi.org/10.3390/app9112377>
- Hossain, M. B., Sultana, N., Noor, P., Khan, S., Lisa, S. A., Begum, M., Punom,

- N. J., Begum, M. K., Hasan, M. R., & Rahman, M. S. (2017). Growth performance and fatty acid profile of Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758) fed with different phytoplankton. *Dhaka University Journal of Biological Sciences*, 26(1). <https://doi.org/10.3329/dujbs.v26i1.46346>
- Ju, Z. Y., Deng, D. F., & Dominy, W. (2012). A defatted microalgae (*Haematococcus pluvialis*) meal as a protein ingredient to partially replace fishmeal in diets of Pacific white shrimp (*Litopenaeus vannamei*, Boone, 1931). *Aquaculture*, 354–355. <https://doi.org/10.1016/j.aquaculture.2012.04.028>
- Kaparapu, J. (2018). Application of Microalgae in Aquaculture. *Phykos*, 48(1), 21–26. <https://www.researchgate.net/publication/342096192>
- Khani, M., Soltani, M., Shamsaie Mehrjan, M., Foroudi, F., & Ghaeni, M. (2017). The effect of *Chlorella vulgaris* (Chlorophyta, Volvocales) microalga on some hematological and immune system parameters of Koi carp (*Cyprinus carpio*). *Iranian Journal of Ichthyology*, 4(1). <https://doi.org/10.22034/iji.v4i1.210>
- Kim, K. W., Bai, S. C., Koo, J. W., Wang, X., & Kim, S. K. (2002). Effects of dietary *Chlorella ellipsoidea* supplementation on growth, blood characteristics, and whole-body composition in juvenile Japanese flounder *Paralichthys olivaceus*. *Journal of the World Aquaculture Society*, 33(4). <https://doi.org/10.1111/j.1749-7345.2002.tb00021.x>
- Li, H., & Liu, J. (2019). Effects of defatted *Haematococcus pluvialis* meal (DHPM) supplementation on the growth performance, and the carotenoid content and composition in the rotifer (*Brachionus plicatilis*). *Aquaculture*, 505. <https://doi.org/10.1016/j.aquaculture.2019.02.027>
- Li, J., Fan, Z., Qu, M., Qiao, X., Sun, J., Bai, D., & CHENG, Z. (2015). *Applications of Microalgae as Feed Additives in Aquaculture*. July. <https://doi.org/10.2991/isesce-15.2015.72>
- Li, M., Wu, W., Zhou, P., Xie, F., Zhou, Q., & Mai, K. (2014). Comparison effect of dietary astaxanthin and *Haematococcus pluvialis* on growth performance, antioxidant status and immune response of large yellow croaker *Pseudosciaena crocea*. *Aquaculture*, 434. <https://doi.org/10.1016/j.aquaculture.2014.08.022>

- Lim, K. C., Yusoff, F. M., Shariff, M., & Kamarudin, M. S. (2018). Astaxanthin as feed supplement in aquatic animals. In *Reviews in Aquaculture* (Vol. 10, Issue 3). <https://doi.org/10.1111/raq.12200>
- Lorenz, R. T., & Cysewski, G. R. (2000). Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. In *Trends in Biotechnology* (Vol. 18, Issue 4). [https://doi.org/10.1016/S0167-7799\(00\)01433-5](https://doi.org/10.1016/S0167-7799(00)01433-5)
- Lulijwa, R., Rupia, E. J., & Alfaro, A. C. (2020). Antibiotic use in aquaculture, policies and regulation, health and environmental risks: a review of the top 15 major producers. *Reviews in Aquaculture*, 12(2), 640–663. <https://doi.org/10.1111/raq.12344>
- Lyons, P. P., Turnbull, J. F., Dawson, K. A., & Crumlish, M. (2017). Effects of low-level dietary microalgae supplementation on the distal intestinal microbiome of farmed rainbow trout *Oncorhynchus mykiss* (Walbaum). *Aquaculture Research*, 48(5). <https://doi.org/10.1111/are.13080>
- Ma, K., Bao, Q., Wu, Y., Chen, S., Zhao, S., Wu, H., & Fan, J. (2020). Evaluation of Microalgae as Immunostimulants and Recombinant Vaccines for Diseases Prevention and Control in Aquaculture. In *Frontiers in Bioengineering and Biotechnology* (Vol. 8). <https://doi.org/10.3389/fbioe.2020.590431>
- Magnadóttir, B. (2006). Innate immunity of fish (overview). *Fish and Shellfish Immunology*, 20(2). <https://doi.org/10.1016/j.fsi.2004.09.006>
- Mahmoud, E. A., El-Sayed, B. M., Mahsoub, Y. H., El-Murr, A. elhakeem I., & Neamat-Allah, A. N. F. (2020). Effect of *Chlorella vulgaris* enriched diet on growth performance, hemato-immunological responses, antioxidant and transcriptomics profile disorders caused by deltamethrin toxicity in Nile tilapia (*Oreochromis niloticus*). *Fish and Shellfish Immunology*, 102, 422–429. <https://doi.org/10.1016/j.fsi.2020.04.061>
- Milan, F. S., Maleki, B. R. S., Moosavy, M. H., Mousavi, S., Sheikhzadeh, N., & Khatibi, S. A. (2021). Ameliorating effects of dietary *Haematococcus pluvialis* on arsenic-induced oxidative stress in rainbow trout (*Oncorhynchus mykiss*) fillet. *Ecotoxicology and Environmental Safety*, 207. <https://doi.org/10.1016/j.ecoenv.2020.111559>
- Mularczyk, M., Michalak, I., & Marycz, K. (2020). Astaxanthin and other nutrients

- from *haematococcus pluvialis*—Multifunctional applications. In *Marine Drugs* (Vol. 18, Issue 9). <https://doi.org/10.3390/md18090459>
- Muller-Feuga, A. (2000). The role of microalgae in aquaculture: situation and trends. *Journal of Applied Phycology*, *12*(3–5), 527–534. <https://doi.org/10.1023/a:1008106304417>
- Ortiz-Chura, A., Pari-Puma, R. M., Rodríguez Huanca, F. H., Cerón-Cucchi, M. E., & Aranibar Aranibar, M. J. (2018). Apparent digestibility of dry matter, organic matter, protein and energy of native Peruvian feedstuffs in juvenile rainbow trout (*Oncorhynchus mykiss*). *Fisheries and Aquatic Sciences*, *21*(1), 1–7. <https://doi.org/10.1186/s41240-018-0111-2>
- Panahi, Y., Khosroshahi, A. Y., Sahebkar, A., & Heidari, H. R. (2019). Impact of cultivation condition and media content on *Chlorella vulgaris* composition. In *Advanced Pharmaceutical Bulletin* (Vol. 9, Issue 2, pp. 182–194). Tabriz University of Medical Sciences. <https://doi.org/10.15171/apb.2019.022>
- Rani, K., Sandal, N., & Sahoo, P. K. (2018). A comprehensive review on chlorella- its composition, health benefits, market, and regulatory scenario. *The Pharma Innovation Journal*, *7*(7), 584–589. www.thepharmajournal.com
- Rasheed, R., Saadaoui, I., Bounnit, T., Cherif, M., Al Ghazal, G., & Al Jabri, H. (2020). Sustainable food production and nutraceutical applications from qatar desert chlorella sp. (*chlorophyceae*). *Animals*, *10*(8), 1–16. <https://doi.org/10.3390/ani10081413>
- Saberi, A., Zorriehzahra, M. J., Emadi, H., Kakoolaki, S., & Fatemi, S. M. R. (2017). Effects of *Chlorella vulgaris* on blood and immunological parameters of Caspian Sea salmon (*Salmo trutta caspius*) Fry exposed to Viral Nervous Necrosis (VNN) virus. *Iranian Journal of Fisheries Sciences*, *16*(2), 494–510.
- Sarker, P. K., Kapuscinski, A. R., Vandenberg, G. W., Proulx, E., & Sitek, A. J. (2020). Towards sustainable and ocean-friendly aquafeeds: Evaluating a fish-free feed for rainbow trout (*Oncorhynchus mykiss*) using three marine microalgae species. In *Elementa* (Vol. 8, Issue 1). <https://doi.org/10.1525/elementa.404>
- Shah, M. M. R., Liang, Y., Cheng, J. J., & Daroch, M. (2016). Astaxanthin-producing green microalga *Haematococcus pluvialis*: From single cell to high

- value commercial products. In *Frontiers in Plant Science* (Vol. 7, Issue APR2016). <https://doi.org/10.3389/fpls.2016.00531>
- Shah, M. R., Lutz, G. A., Alam, A., Sarker, P., Kabir Chowdhury, M. A., Parsaemehr, A., Liang, Y., & Daroch, M. (2018). Microalgae in aquafeeds for a sustainable aquaculture industry. *Journal of Applied Phycology*, 30(1). <https://doi.org/10.1007/s10811-017-1234-z>
- Sheikhzadeh, N., Tayefi-Nasrabadi, H., Oushani, A. K., & Enferadi, M. H. N. (2012). Effects of *Haematococcus Pluvialis* Supplementation on Antioxidant System and Metabolism in Rainbow Trout (*Oncorhynchus Mykiss*). *Fish Physiology and Biochemistry*, 38(2). <https://doi.org/10.1007/s10695-011-9519-7>
- Shi, X., Luo, Z., Chen, F., Wei, C. C., Wu, K., Zhu, X. M., & Liu, X. (2017). Effect of fish meal replacement by Chlorella meal with dietary cellulase addition on growth performance, digestive enzymatic activities, histology and myogenic genes' expression for crucian carp *Carassius auratus*. *Aquaculture Research*, 48(6), 3244–3256. <https://doi.org/10.1111/are.13154>
- Sirakov, I., Velichkova, K., Stoyanova, S., & Staykov, Y. (2015). The importance of microalgae for aquaculture industry . Review . *International Journal of Fisheries and Aquatic Studies*, 2(4), 81–84. <http://www.fisheriesjournal.com/archives/2015/vol2issue4/PartB/2-4-31.pdf>
- Skalli, A., Firmino, J. P., Andree, K. B., Salomón, R., Estévez, A., Puig, P., Sabater-Martínez, M., Hechavarria, T., & Gisbert, E. (2020). The inclusion of the microalga *scenedesmus sp.* In diets for rainbow trout, *onchorhynchus mykiss*, juveniles. *Animals*, 10(9). <https://doi.org/10.3390/ani10091656>
- Taghavi Takyar, M. B., Haghghat Khajavi, S., & Safari, R. (2019). Evaluation of antioxidant properties of *Chlorella vulgaris* and *Spirulina platensis* and their application in order to extend the shelf life of rainbow trout (*Oncorhynchus mykiss*) fillets during refrigerated storage. *LWT*, 100. <https://doi.org/10.1016/j.lwt.2018.10.079>
- Thépot, V., Campbell, A. H., Rimmer, M. A., & Paul, N. A. (2020). Meta-analysis of the use of seaweeds and their extracts as immunostimulants for fish: a systematic review. In *Reviews in Aquaculture*. <https://doi.org/10.1111/raq.12504>

- Tibbetts, S. M., Scaife, M. A., & Armenta, R. E. (2020). Apparent digestibility of proximate nutrients, energy and fatty acids in nutritionally-balanced diets with partial or complete replacement of dietary fish oil with microbial oil from a novel *Schizochytrium sp.* (T18) by juvenile Atlantic salmon (*Salmo salar L.*). *Aquaculture*, 520. <https://doi.org/10.1016/j.aquaculture.2020.735003>
- Vallejos-Vidal, E., Reyes-López, F., Teles, M., & MacKenzie, S. (2016). The response of fish to immunostimulant diets. In *Fish and Shellfish Immunology* (Vol. 56). <https://doi.org/10.1016/j.fsi.2016.06.028>
- Xie, J., Fang, H., He, X., Liao, S., Liu, Y., Tian, L., & Niu, J. (2020). Study on mechanism of synthetic astaxanthin and *Haematococcus pluvialis* improving the growth performance and antioxidant capacity under acute hypoxia stress of golden pompano (*Trachinotus ovatus*) and enhancing anti-inflammatory by activating Nrf2-ARE pathway to antagonize the NF-κB pathway. *Aquaculture*, 518. <https://doi.org/10.1016/j.aquaculture.2019.734657>
- Xie, J., Fang, H., Liao, S., Guo, T., Yin, P., Liu, Y., Tian, L., & Niu, J. (2019). Study on *Schizochytrium sp.* improving the growth performance and non-specific immunity of golden pompano (*Trachinotus ovatus*) while not affecting the antioxidant capacity. *Fish and Shellfish Immunology*, 95. <https://doi.org/10.1016/j.fsi.2019.10.028>
- Xie, S., Fang, W., Wei, D., Liu, Y., Yin, P., Niu, J., & Tian, L. (2018). Dietary supplementation of *Haematococcus pluvialis* improved the immune capacity and low salinity tolerance ability of post-larval white shrimp, *Litopenaeus vannamei*. *Fish and Shellfish Immunology*, 80. <https://doi.org/10.1016/j.fsi.2018.06.039>
- Xu, W., Gao, Z., Qi, Z., Qiu, M., Peng, J. Q., & Shao, R. (2014). Effect of dietary chlorella on the growth performance and physiological parameters of gibel carp, *Carassius auratus gibelio*. *Turkish Journal of Fisheries and Aquatic Sciences*, 14(1). https://doi.org/10.4194/1303-2712-v14_1_07
- Yarnold, J., Karan, H., Oey, M., & Hankamer, B. (2019). Microalgal Aquafeeds As Part of a Circular Bioeconomy. In *Trends in Plant Science* (Vol. 24, Issue 10). <https://doi.org/10.1016/j.tplants.2019.06.005>
- Yu, W., Lin, H., Yang, Y., Zhou, Q., Chen, H., Huang, X., Zhou, C., Huang, Z., & Li, T. (2020). Effects of supplemental dietary *Haematococcus pluvialis* on

growth performance, antioxidant capacity, immune responses, and resistance to *Vibrio harveyi* challenge of spotted sea bass *Lateolabrax maculatus*. *Aquaculture Nutrition*. <https://doi.org/10.1111/anu.13189>

Yuan, J., Ni, M., Liu, M., Zheng, Y., & Gu, Z. (2019). Occurrence of antibiotics and antibiotic resistance genes in a typical estuary aquaculture region of Hangzhou Bay, China. *Marine Pollution Bulletin*, 138. <https://doi.org/10.1016/j.marpolbul.2018.11.037>

Zhang, F., Man, Y. B., Mo, W. Y., & Wong, M. H. (2020). Application of Spirulina in aquaculture: a review on wastewater treatment and fish growth. In *Reviews in Aquaculture* (Vol. 12, Issue 2). <https://doi.org/10.1111/raq.12341>

Zhao, Y., Yang, Q. E., Zhou, X., Wang, F. H., Muurinen, J., Virta, M. P., Brandt, K. K., & Zhu, Y. G. (2020). Antibiotic resistome in the livestock and aquaculture industries: Status and solutions. *Critical Reviews in Environmental Science and Technology*, 0(0), 1–38. <https://doi.org/10.1080/10643389.2020.1777815>

Zhou, M., Yu, S., Hong, B., Li, J., Han, H., & Qie, G. (2021). Antibiotics control in aquaculture requires more than antibiotic-free feeds: A tilapia farming case. *Environmental Pollution*, 268, 115854. <https://doi.org/10.1016/j.envpol.2020.115854>

VI. Appendix

1. Growth performances and survival of rainbow trout fed the experimental diets for 6 weeks

Formula	Tank No	WG	SGR	FE	PER	CF	HIS	VSI	SUR
CONT	5	85.6	1.72	74.9	1.46	1.01	1.08	4.25	93.3
	7	92.3	1.82	74.5	1.45	1.10	1.27	5.69	86.7
	24	71.4	1.50	70.4	1.37	0.95	1.07	4.28	100
CHLO	6	72.8	1.52	61.2	1.18	1.02	1.32	5.69	80.0
	19	81.9	1.66	72.5	1.40	1.01	1.06	4.15	93.3
	26	80.2	1.64	71.4	1.38	1.00	1.21	4.37	86.7
HAEM	9	104	1.98	74.5	1.49	0.96	1.11	4.14	80.0
	11	96.8	1.88	71.6	1.44	1.00	1.11	4.97	80.0
	21	97.5	1.89	72.4	1.45	1.06	1.07	5.22	93.3
SCHIZO	8	65.7	1.40	59.8	1.19	1.00	1.39	6.09	93.3
	10	76.9	1.58	71.4	1.42	0.93	0.95	3.68	100
	20	71.2	1.49	64.6	1.28	1.04	1.17	4.65	93.3

Diets represent CONT = Control, CLHO = *Chlorella sp.*, HAEM = *Haematococcus sp.*, SCHIZO = *Schizochytrium sp.*

2. Biochemical parameters and non-specific immune responses of rainbow trout fed the experimental diets for 6 weeks

Formula	Tank No	AST	ALT	GLU	TP	MPO	LYZ
CONT	5	36.1	10.0	89.0	3.60	1.29	1.82
	7	43.5	7.0	77.0	3.30	1.18	2.05
	24	35.5	9.0	81.0	3.50	1.04	2.40
CHLO	6	46.7	9.0	75.0	3.50	0.95	1.80
	19	38.5	8.0	76.0	3.90	1.43	1.97
	26	45.3	9.0	70.0	3.70	1.15	1.88
HAEM	9	36.8	5.0	85.0	3.50	1.51	6.90
	11	38.1	6.0	82.0	3.70	1.08	6.22
	21	62.0	8.0	83.0	3.90	1.29	2.12
SCHIZO	8	55.1	8.0	73.0	3.30	1.25	2.13
	10	56.6	9.0	69.0	3.40	1.22	2.05
	20	46.9	7.0	69.0	3.50	1.21	1.78

Diets represent CONT = Control, CLHO = *Chlorella sp.*, HAEM = *Haematococcus sp.*, SCHIZO = *Schizochytrium sp.*