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INTEGRATION OF SPLEEN ^{1}H NMR-BASED METABOLOMICS REVEALED THE IMMUNOMODULATORY EFFECT OF *Chlorella vulgaris* ON NILE TILAPIA (*Oreochromis niloticus*)

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ABSTRACT

Tilapia (*Oreochromis* sp.) is among the major aquaculture fish species that has a high market value for both local consumption and global export. However, emergence of viral, bacterial, and fungal diseases has become a major threat to its production. New feed materials that can enhance its immunity against such threats are therefore much needed. The present study investigated the immunomodulatory effect of microalgae (*Chlorella vulgaris)* on *Oreochromis niloticus*, as the fish model. Groups of tilapia were fed with commercial feed supplemented with four different concentrations (62.5, 125, 250 and 500 mg kg^{-1} body weight) of *C. vulgaris* over a period of twenty-one days, following which ^{1}H NMR-based metabolomics was applied to identify the potential immunomodulation biomarkers from metabolic changes observed in the fish spleen. Prior to the feeding experiment, acute toxicity assay was also carried out, which indicated that up to a concentration of 500 mg/kg body weight, *C. vulgaris* is not toxic to the fish model. The four *C. vulgaris*-supplemented experimental diets resulted in improvements in the immune response (phagocytosis, respiratory burst activity and lymphoproliferation) and metabolomics analysis of the modulations observed in the spleen ^{1}H NMR profiles revealed that amino acid, fatty acid and riboflavin metabolisms were involved in the immune response. Based on their up-regulations, riboflavin, choline, stearic acid, linoleic acid, linolenic acid and taurine were identified as some of the key biomarkers. This study reports for the first time the biomarkers and the possible metabolic pathways associated with the immune enhancing effect of *C. vulgaris*. The results of the study support the high potential that the microalgae has in terms of being an immune enhancing supplement to conventional feed material for use by the aquaculture industry.

Keywords: *Chlorella vulgaris*; *Oreochromis niloticus*; immunomodulation; metabolic pathway; ^{1}H NMR-based metabolomics.

INTRODUCTION

Aquaculture production is playing an increasingly important role in meeting the demand for fish and fishery products for human consumption. Presently, one third of the total food fish supply is derived from aquaculture, while the remaining two-thirds are obtained from capture fisheries in marine and inland water (WHO, 2020). In aquaculture, tilapia (*Oreochromis* sp.) is among the important fish species, favoured due to its exceptionally fast growth rate and high tolerance towards various harsh environmental conditions (Zahran & Risha,

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2014). The overall world production of tilapia is expected to increase as a result of their outstanding features, thereby making it a profitable protein source (FAO, 2017). The world production of tilapia in 2019 is about 6.5 million metric tonnes (MT) , which amounted to an estimated value of USD 12 billion, with China and Indonesia leading the list of main tilapia-producing countries (FAO, 2019). Tilapia is the second most harvested freshwater fish in Malaysia, amounting to a total production of 33,437 tonnes per year (FAO, 2019). This is estimated to be a wholesale value of RM 259 million per annum, which emphasizes the importance of tilapia farming in Malaysia (AFS, 2013). However, the sustainability of tilapia aquaculture faces some constraints since tilapia is prone to infections caused by viruses, bacteria, fungi and parasites (Giri et al., 2012), in addition to other factors such as nutritional imbalances, environmental conditions and handling stress (Ahmad et al., 2018). In Malaysia, tilapia culture is commonly affected by *Aeromonas hydrophila* and *Streptococcus agalactiae* (Amal et al., 2018). There has also been recent reports on the emergence of Tilapia Lake Virus (TiLV) (Amal et al., 2018). The latter is a novel RNA virus, responsible for massive deaths of cultured tilapia fish in Israel, Ecuador, Colombia and Egypt (Eyngor et al., 2014; Ferguson et al., 2014; Fathi et al., 2017; Tsofack et al., 2017). The virus also caused high mortality of cultured red tilapia fish (*O. aureus* x *O. mossambicus*) and Nile tilapia fish (*O. niloticus*) in Thailand (Dong et al., 2017a; Dong et al., 2017b; Surachetpong et al., 2017). Problems such as these will greatly affect fish production and consequently will directly pose negative economic impacts to the aquaculture industry as a whole (Ismail et al., 2016). Over reliance on antibiotics to control or reduce disease infections has led to contamination of water bodies and development of drug resistance (Schoepp et al., 2015), as well as toxicity issues in downstream fish products (Jazrawi et al., 2015). Although the use of vaccines and probiotics has helped curb the problem to a certain extent, other more effective solutions are still much needed. Using microalgae-based feed as immunostimulants to enhance fish immunity against bacterial infections offers a promising alternative that will complement other modalities used in fish disease management.

High value microalgae species produce an array of compounds of various classes that can potentially contribute to immune improvements of living organisms. In this regard, pharmacological potential and economic affordability of microalgae-derived drugs have gain prominence and garnered increased research interests (Markou & Nerantzis, 2013; Chen et al., 2016). From the perspective of food and feed materials, microalgae can be considered as an innovative and promising food ingredient, rich in nutrients such as high value proteins, polyunsaturated fatty acids (PUFAs), carotenoids, vitamins, minerals, chlorophylls and phenolic compounds, as well as other bioactive molecules (Batista et al., 2017). Microalgal biomass have been integrated with food items such as pasta, biscuits and vegetarian desserts, in developing innovative and healthy food products (Batista et al., 2012). Similarly, supplementing fish diets with microalgae biomass is expected to bring more nutritive and health benefits. Past researches on the utilisation of microalgae as immunostimulants, suggested that suitable amounts to be incorporated into fish feed are in the range of 12 to 30% for a feeding period of 60 to 90 days (Ahmad et al., 2018). In practice, although the cost of producing microalgae is considerably high and digestibility of some microalgae species may be a compounding factor, incorporating small amounts of microalgal biomass in fish feed could still be

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economically feasible in aquaculture. Digestibility problems could be solved if immune enhancement can be achieved with lower microalgal concentrations supplemented into the fish feed.

Chlorella vulgaris is a high value freshwater green microalga, belonging to the family *Chlorellaceae* of the division Chlorophyta (Safi et al., 2014). Presently, *C. vulgaris* is well known as a health food or supplement, valued for its high chlorophyll, carotenoid, and protein contents (Sharma et al., 2011). *Chlorella vulgaris* synthesizes all the essential amino acids as well as some of the non-essential amino acids, and has a better profile than the standards proposed for human nutrition by Food and Agricultural Organization (FAO) and World Health Organization (WHO) (Safi et al., 2014b). These nutritive characteristics gave the spherical, single-celled microalgae an added advantage over other microalgal species. Past research also reported that *C. vulgaris* is an excellent source of fatty acids due to its high lipid content (Varfolomeev & Wasserman, 2011). It has been reported that supplementing fish diets with *C. vulgaris* increases prophenoloxidase activity, total hemocyte and resistance against *Macrobrachium rosenbergii* post larvae and *Aeromonas hydrophila* infections (Maliwat et al., 2016). In addition, increased growth performance and total fish protein content were both observed when using fish feed supplemented with *C. vulgaris* (Maliwat et al., 2016). Whilst the final outcomes of these interventions were successfully shown, pertinent questions as to what the metabolic perturbations and mechanism or mode of action are involved in the manifestations of these effects, were largely left unanswered and not further evaluated. Although the bioactive properties of microalgae biomass have been extensively demonstrated, there is no information available regarding the biomarkers for immune improvement associated with *C. vulgaris*. Thus, the exact way *C. vulgaris* impacted immunogenesis in the biological system has yet to be fully understood.

Metabolomics, which is profiling of low-molecular weight metabolic entities (usually < 1000 Da) within a cell, tissue, organ, biological fluid or the entire organism (Webb-Robertson et al., 2005), are now routinely applied as a tool to identify biomarkers indicative of a specific condition or a particular metabolic pathway. Owing to innovative developments in informatics and analytical technologies, and the integration of orthogonal biological approaches, it is now possible to expand metabolomic analyses to understand the systems-level effects of metabolites. Moreover, because of the inherent sensitivity of metabolomics, subtle alterations in biological pathways can be detected to provide insights into the mechanisms underlying various physiological conditions and processes (Kaddurah-Daouk et al., 2008; Sethi & Brietzke, 2016), for example an organism's response to external stimuli such as diseases, contaminants, nutritional imbalance and immune stimulants (Lankadurai et al., 2013). Application of metabolomics in aquaculture research, although quite recent, has produced interesting results and furthered the understanding of the metabolic signatures and interplay between cause-effects of the various biological processes involved. For example, serum metabolomics was employed in combination with transcriptomics, in a study on the immune-responses and metabolic changes of the tapertail anchovy (*Coilia nasus*) infected by Anisakidae parasite, which provided deeper insights into the population ecology of the estuarine species (Liu et al., 2019). Meanwhile, Jiao et al., (2020) also employed serum metabolomics to understand the physiological regulation in response to cold and starvation stresses, in yellow drum (*Nibea albiflora*) where they

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found that glutathione metabolism and its related metabolites (glutamate and oxidized glutathione) could be the important biomarkers.

In the present study, the effect of supplementing fish diet with *C. vulgaris* was investigated by assessing the innate immune responses of tilapia (*O. niloticus*) fed with experimental diets supplemented with different concentrations of the microalgae. Additionally, spleen ^{1}H NMR-based metabolomics was employed to obtain further insights into the immunomodulatory effects of *C. vulgaris* on tilapia (*O. niloticus*), the key biomarkers and pathways involved in the immune response.

MATERIALS AND METHODS

Fish Maintenance

Juvenile *O. niloticus* with average individual weights of 70 ± 0.5 g (mean ± SE) were obtained from Taman Pertanian Universiti (TPU), Universiti Putra Malaysia (UPM). Upon transportation to the fish hatchery of Laboratory of Marine Biotechnology (MARSLAB), Institute of Bioscience (IBS), UPM, the fishes were placed in glass aquaria (80x40x30 cm) and acclimatized for 2 weeks, fed twice daily (morning and evening) with commercial basal diet, each time at 0.5% body weight (bw). The holding water (dechlorinated) temperature was maintained at 25-28°C, while pH and dissolved oxygen at 6.9-7.3 and 9-12 mg/L, respectively (Gilcreas, 1966). All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University, with the approval reference number (UPM/IACUC/AUP-R080/2018).

Chlorella vulgaris Biomass Culture

Starter-culture of *C. vulgaris* was obtained from the aquatic laboratory of the Faculty of Veterinary Medicine, UPM. The identity of *C. vulgaris* 'was authenticated by experts in Aquatic Laboratory Faculty of Veterinary Medicine, UPM Serdang and further morphological characteristics were confirmed by 400X microscopic view. The culture medium was prepared by raising 200 L of cultured media from 40 mL of starter-culture using Bold Basal's culture media (BBM). The whole process was conducted in duplicate (100 L x 2) and lasted for two months *via* successive addition of 10 L of culturing media in three days interval after the growth is checked using hemocytometer under a 400X microscope. The parameter for the algal culture was adopted from the original BBM media. The microalgae biomass product was cultivated in fresh water containing the prepared BBM media having various concentrations of different acids and autoclaved trace elements solution. The growth medium was adjusted and monitored at pH 7.5. The wet crude product was harvested via centrifugation by employing high-speed Sorvall Evolution RC centrifuge (Thermo Electron Corporation, Asheville, NC, USA) at 12,000 rpm at 25^{o}C. It was then kept at -80^{o}C for five days and lyophilized using freeze drier. The obtained biomass was then ground into fine powder using a 500 mL tumbler blender (Panasonic, Model No. PSN-MXGM0501). The culture medium upscale parameters (BBM) for growing freshwater *C. vulgaris* microalgae sample are shown in Table S1 of the supplementary data (Pantami etal., 2021).

Preparation of *C. vulgaris*-supplemented Feed Material

The microalgae-supplemented feed was prepared by mixing commercial fish feed (Aqua Feed Additives, Malaysia) with various proportions of cultured *C. vulgaris* biomass (0.625%, 1.25%, 2.5% and 5%). The modified feed was produced by re-pelleting using syringe. Re-pelleting was carried out by passing the wet microalgae-supplemented feed through a 10 ml syringe and cutting the extruded material into 2 mm

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length. Feed pellets were then oven-dried at 40^{o}C before use. The prepared feed pellets were observed to float freely on the surface of the water and were well accepted by the fish. Proximate analysis was performed on the various *C. vulgaris*-supplemented feed, the results of which are shown in Table S2 of the supplementary data. The results of the analysis indicated that all the prepared feed satisfied the minimal nutrient content requirements for fish feed (Bhuiyan et al., 2016; Kumar et al., 2015).

Experimental Design

The study was conducted in two phases, comprising i) determining a safe concentration of *C. vulgaris*, and ii) evaluating the effect of *C. vulgaris*-supplemented diet on *O. niloticus*. For the first phase, fish were divided into two groups of 6, to represent control and treatment group, respectively. The treatment group was fed, via oral gavage, with 0.2 mL of 500 mgkg^{-1} bw of *C. vulgaris* biomass suspended in 0.1% phosphate buffer saline (PBS), while the control group was fed with 0.2 mL of 0.1% PBS only. Both groups were monitored over a fasting period of 4 days, after which, all fish were anesthetized using MS-222 anaesthesia (Shanghai Reagent Corp., Shanghai, China) (10 ml per 2 L of water) for 5 minutes and sacrificed. Blood samples were taken from the caudal vein of each fish and allowed to coagulate on ice for 30 minutes. The serum was separated by centrifugation at 1800 rpm and subjected to biochemical analysis. Fish liver and gill were also sampled for histopathological analysis.

For the second phase of the experiment, fish were divided into 5 groups. To ensure 6 replications for each observation, each group were allocated 12 fish, paired into 6 so that n=6 for each group. In the case where a fish died, the duplicate was used in its place. The groups were labelled as the control group A (commercial feed only), and treatment groups B, C, D and E, for the respective experimental *C. vulgaris*-supplemented diets (Table 1). The distribution of fish into the 5 groups and arrangement of the replicates (1 to 6) in each group followed completely randomized design (CRD) (Kwak et al., 2012) to avoid data biasness which could arise from differing environmental conditions. To ensure that each fish received the desired amount of feed meant for the group, one aquarium was occupied by only two fishes, separated using a plastic mesh. Fish were fed with the respective diets over 21 days.

After 3 weeks, fish were sacrificed by immersing them in ice water for 15 minutes (Mediani et al., 2016), upon which the spleen was dissected from the representative fish of each pair. Each spleen sample was divided into 2 parts, one part for ^{1}H NMR metabolomics analysis and the other for immunological evaluation (phagocytosis, respiratory burst, and lymphoproliferation activities). For the former, samples were immediately quenched with liquid nitrogen, crushed into powder, and kept at -80^{o}C prior to spectral measurement, while for the latter, samples were kept at -80^{o}C in freezing media until required. The freezing media was prepared by mixing 10 % DMSO with 90 % complete media comprising of 10 % fetal bovine serum (FBS) in 90 % Dulbecco′s Modified Eagle′s Medium (DMEM). Both procedures were performed under sterile conditions in a laminar flow. Prior to immunological analysis, the frozen samples were thawed and washed 3 times using 10 mL cold 1% PBS.

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Table 1. Distribution of fish for feeding experiment.

Group	Fish number*	Proportion of microalgae in fish diet
A (control)	A1, A2, A3, A4, A5, A6	0 $mgkg^{-1}$ bw (0%)
B	B1, B2, B3, B4, B5, B6	62.5 $mgkg^{-1}$ bw (0.625 %)
C	C1, C2, C3, C4, C5, C6	125 $mgkg^{-1}$ bw (1.25 %)
D	D1, D2, D3, D4, D5, D6	250 $mgkg^{-1}$ bw (2.5 %)
E	E1, E2, E3, E4, E5, E6	500 $mgkg^{-1}$ bw (5 %)

*Each group comprised of 6 pairs of fish, labelled 1 to 6 according to the group label (A to E)

Phagocytosis Activity Assay

Phagocytic activity (expressed as % phagocytic cells) was conducted according to the method described by Selim & Reda (2015), using leucocytes suspension prepared from the fish spleen (Selim & Reda, 2015). The activity was measured based on the particulate uptake property of the lymphoid cells (Anderson & Swicki, 1995). In the present study, yeast cell was used as the particulate cells. Equal volumes (100 L) of spleen and instant dry yeast (Afaweez Groups, Kuala Lumpur, Malaysia) cell suspensions were mixed well together by pipetting several times and incubated for 20 min at 25°C. After incubation, 6 µL of the mixture was spread onto a clean microscope slide, precoated with 10% Poly L-Lysine solution (Sigma-Aldrich, USA), to make a thin and uniform smear. The prepared slides were airdried and fixed with methanol (1 min) followed by May-Grunwald solution (5 min) before finally staining with 7% Giemsa (20 min). The stained slides were gently rinsed with water and airdried. One hundred cells were counted at various parts of the slide, viewed at 100X digital microscopic (Unitron, Chicago, USA) magnification. The percentage of phagocytic cells was then determined as follows:

$$\% \text{ phagocytic cells} = \frac{\text{Number of phagocytic cells}}{\text{Number of nonphagocytic cells}} \times 100$$

Respiratory burst activity assay

Leukocyte respiratory burst activity (RBA) was determined using the method of Sirimanapong et al (2015) with slight modifications. Briefly, 100 µL of spleen cell suspension ($1x10^6$ cells mL^{-1}) was mixed with an equal volume of 5 $mgmL^{-1}$ zymosan (Sigma-Aldrich, USA) as the foreign pathogen. The mixture was incubated for 30 min at 37°C, and then centrifuged at 800 rpm. The supernatant was discarded, and the leucocytes washed three times with distilled water. To determine the superoxide anion generated, the leucocytes were then incubated with 100 µL of 2 $mgmL^{-1}$nitroblue tetrazolium solution (Sigma-Aldrich, USA) for 30 min, after which the reaction was terminated by adding 100 µL methanol. After centrifuging, the formazan crystals formed were dissolved in a mixture of 120 µL of 2M potassium hydroxide and 140 µL dimethyl sulfoxide. The optical density (OD) of the resulting colored solution was then measured at 630 nm, using ELISA reader (AgileReader™, USA), to give the stimulated activity (SA) value. For basal or spontaneous activity (BA) value, the same procedure was repeated, but without zymosan. The respiratory burst activity (RBA) was then calculated according to the formula:

RBA = Stimulated activity (SA) - Basal activity (BA).

Where SA = the RBA caused by stimulation with zymosan, and BA = the spontaneous RBA (without zymosan).

Lymphoproliferation Activity Assay

The method of Dupont et al (2013) with slight modifications was adopted for measurement of lymphoproliferation activity. Different mitogens were used to assess the activation of T-cells and B-cells. For the former, 10 $mgmL^{-1}$ of

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phytohemaglutinin (Sigma-Aldrich, USA) was used, while 40 $mgmL^{-1}$ lipopolysaccharide (Sigma-Aldrich, USA) was used for the latter. Briefly, 100 µL of spleen cell suspension ($1x10^5$ cells mL^{-1}) was added and mixed well with an equal volume of the specific mitogen, and incubated in a 5% CO_2 incubator (ESCO, CCL-170B-8, Changi Singapore) at 25°C for 48 hours. The resulting cells were dispersed in 1 mL of 0.1% PBS and counted. The same procedure was repeated on another 100 µL of spleen cell suspension but without mitogen. This later value (without mitogen) was then subtracted from the mitogen-stimulated proliferation value to get the difference as the number of mitogen stimulated cells. Lymphoproliferation activity was expressed as stimulation index (SI), calculated as the ratio of proliferation of mitogen-induced stimulated cells to that of cells incubated without mitogen (Dupont et al., 2013), using the formula:

$$SI = \frac{\text{Number of mitogen stimulated cells}}{\text{Number of corresponding control cells}}$$

^{1}H NMR spectral measurements

Fish spleen (15 mg) was mixed with 600 µL of 0.5M Na_2HPO_4 and 0.1% TSP (dissolved in D_2O, pH 6.0). The mixture was homogenized by vortexing for 2 min and centrifuged at 10,000 rpm for 10 minutes at room temperature. The clear supernatant (500 µL) was transferred into 5mm NMR tube (Norell, USA) for spectral acquisition.

NMR spectral measurements for metabolite identification were conducted on a Bruker Ascend 700MHz NMR spectrometer equipped with a TCI Cryo-Probe (Bruker Biospin, Billerica, Massachusetts, USA). ^{1}H NMR data acquisition was acquired at 25^{o}C, using a single-pulse proton experiment (with PRESAT) using 21.0 µs pulse width, 2 sec relaxation delay, 4.0 min total acquisition time, and 64 scans. *J*-resolved experiment was carried out for additional support of spectral assignments. The *J*-resolved spectrum acquisition time was 48 min and 30 sec, with 16 scans per 300 increments for the axis of the spin-spin coupling constant (*J*) and spectral widths of 66 Hz, and 8 K data points for the chemical shift axis with spectral width of 9012.8 Hz. Relaxation delay time was set to 1.0 sec.

Two-dimensional heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond coherence (HMBC) spectra were obtained using 16 scans, 1 K data points, and 300 increments at the spectral width of 13 ppm and 220 ppm for the proton and carbon dimensions respectively. The relaxation delay time was set to 1.0 sec, giving a total time of 7 h, 35 min, and 45 sec. The two-dimensional NMR spectral processing and analysis for structural elucidation were carried out using MestReNova version 6.02-5475 (Mestrelab Research, Santiago de Compostella, Spain) and Chenomx NMR Suite version 8.31 (Chenomx Inc., Edmonton, Canada).

Spectral Data Preprocessing and Multivariate Analysis

^{1}H NMR spectra were automatically Fourier-transformed to ASCII (American Standard Code for Information Interchange) files, phased and baseline-corrected before referenced to the internal standard using Chenomx software (version 6.2, Edmonton, Alberta, Canada). TSP was used as a reference internal standard, set at 0.0 ppm. After excluding the spectral region between 4.60 to 4.88 ppm (residual water signal), the ^{1}H NMR spectra (0.50 to 10.00 ppm) were divided into bins of 0.04 ppm width. The standardized binned data was then pareto-scaled and subjected to partial least square discriminant analysis (PLS-DA) and partial least squares (PLS) regression analysis, using SIMCA-P+ software (version 12.0.1.0, Umetrics AB, Umea, Sweden. In the generated score plot, each

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variable was represented as an individual spleen sample, which allowed the observation of the classification behaviour of spleen samples along two main principal components, PC1 and PC2. The metabolites responsible for the groups' separation were identified based on the variables that appeared in the loading plots, represented as individual bins of the ^{1}H NMR data. Validation and fitting of the generated models were carried out using three-fold approach comprising 100 permutation tests, computation of R2Y, Q2Y, and CV-ANOVA values (Verpoorte et al., 2007).

Statistical Analysis

Statistical analysis was performed using SPSS version 16 (SPSS Inc., Michigan Avenue, Chicago, IL, USA). One-way analysis of variance (ANOVA) with Tukey's post hoc test was used to determine the significance differences within and across groups, at a confidence level of 95%.

RESULTS AND DISCUSSION

Safe Concentration of *Chlorella vulgaris* Biomass

The results of serum biochemical analysis of fish fed with 500 mgkg^{-1} bw of *C. vulgaris* biomass. No fish mortality was recorded during the experiment. All nine replicates of fish in both control and microalgae-fed groups swam normally at the same speed rate. For each of the biochemical parameters evaluated (aspartate aminotransferase, total protein, albumin, globulin, albumin:globulin ratio, and alanine aminotransferase), there were no significant differences between the concentrations recorded for the microalgae-fed fish and control groups. These results indicated that a dose of 500 mgkg^{-1} bw *C. vulgaris* was non-toxic to the fish. In addition, morphological analysis of the organs of fish fed with *C. vulgaris* showed no alterations after treatment, which conformed to the biochemical test results.

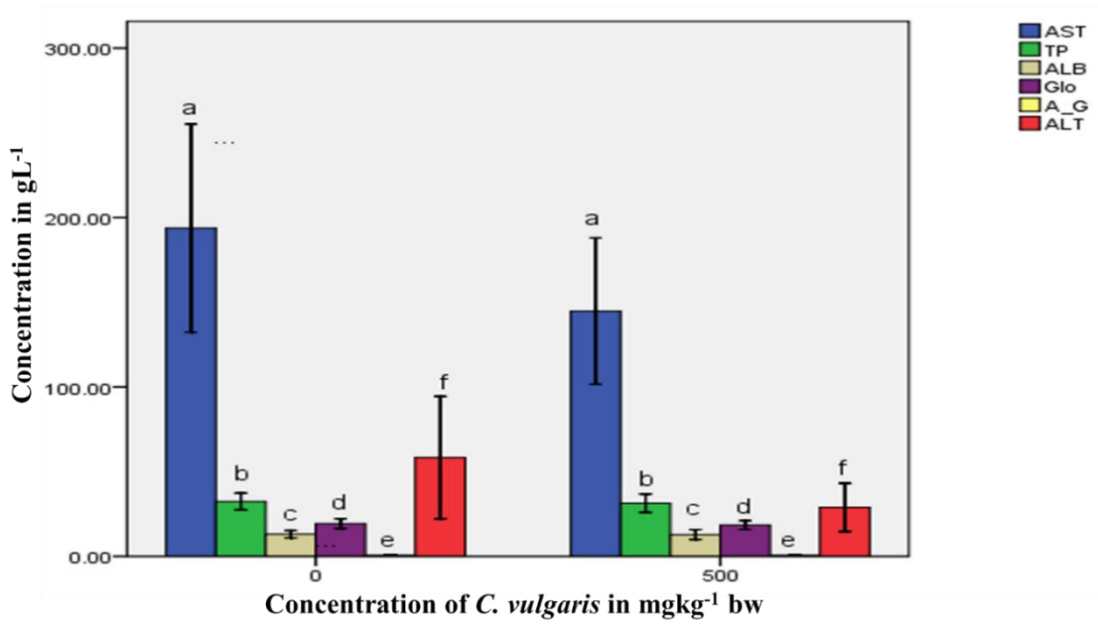

Figure 1: Results of biochemical analysis of fish serum from microalgae-fed (500 mgkg^{-1} bw *Chlorella vulgaris* in 0.1% PBS) and control (0.1% PBS) groups. AST (aspartate aminotransferase), TP (total protein), ALB (albumin), Glo (Globulin), A:G (albumin:globulin ratio), ALT (alanine aminotransferase). Data was expressed as mean±SE (n=9). Mean values bearing the same letters are not significantly different at $p > 0.05$.

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Histopathological examination was also conducted on fish liver and gills. Microscopic images (magnification X100) of the biological tissues are provided as supplementary materials (Figure S2). In general, there were no observable differences in the histopathological profiles of the organs obtained from both groups of fish. In addition, there were no observable signs of lesion in the organs obtained from the microalgae-fed group. Gill samples of the microalgae-fed group showed normal characteristic arrangement. The gills comprised of four sets of gill lamellae and both sides were reinforced by bony structure and primary lamellae. The secondary lamellae comprised of several blood capillaries, which were segmented by single layered pillar cells. There was also abundance of mucous cells on the epithelial gill rackers. In addition, the primary lamellae had relatively smaller and lesser number of mucous cells (Aldoghachi et al., 2016).

Liver samples of the microalgae-fed group showed the normal, typically compact structure, where the hepatocytes presented normal characteristic cytoplasmic distribution and nuclear morphology (Aldoghachi et al., 2016). Observation of the liver sections revealed that the liver exhibited normal architecture, and no pathological abnormalities were detected. The hepatocytes had a homogeneous cytoplasm and large central or sub-central spherical nucleus and densely stained nucleolus (Figure S1) (Alm-Eldeen et al., 2018).

Effect of *Chlorella vulgaris*-Supplemented Diet on Innate Immune Responses of Tilapia

Phagocytosis Activity

Phagocytosis activity indicates the ability of the lymphoid cells of the fish spleen to engulf foreign particles that could be potentially toxic to the fish (Selim & Reda, 2015). The extent to which the cells demonstrated phagocytosis activity was compared in terms of percentage phagocytic cells for every 100 cells counted in each sample smear. As shown in Figure 2(A), phagocytic activity of all groups fed with *C. vulgaris*-supplemented feed was significantly higher than the control and significantly different between them although the trend was not dose-dependent. Group C (feed supplemented with 125 $mgkg^{-1}$ bw *C. vulgaris*) exhibited significantly high phagocytic activity, with the highest increment of 60%. This was followed by group E (feed supplemented with 500 $mgkg^{-1}$ bw *C. vulgaris*) with 28% increment, while groups B and D (feed supplemented with 62.5 $mgkg^{-1}$ bw and 250 $mgkg^{-1}$ bw *C. vulgaris*, respectively) displayed lower activities, recording 10-12% increment. Phagocytic and non-phagocytic cells are shown as supplementary files (Figure S3). These results indicated that the 3 weeks treatment with *C. vulgaris* have significantly improved the fish spleen phagocytosis activity. In addition, the results contrasted with the findings of Teuling et al., (2017) in which no noticeable effects on overall phagocytic activity in tilapia fish were recorded despite the 300 g kg^{-1} bw *C. vulgaris* biomass inclusion level. This may be attributed to the indigestibility issue of *C. vulgaris* when consumed in higher doses and without rapturing the cell walls (Ahmad et al., 2018).

Respiratory burst activity

Respiratory burst activity indicates the ability of the lymphoid organ cell suspension in destroying an engulfed pathogen (Dad & Ikhwanuddin, 2012). As shown in Figure 2(B), compared to the control, the pathogen destroying capacity was significantly increased for groups fed with feed supplemented with high contents of *C. vulgaris*, although not in a dose-

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dependent manner. The activity was highest for group D (250 mgkg^{-1} bw) which exhibited 50% increment in activity, followed by group E (500 mgkg^{-1} bw) with 28.5% increment. The capacity of groups fed with feed supplemented with lower contents of *C. vulgaris* (groups B and C), were not significantly different with the control group. The results also indicated that 3 weeks feeding with the *C. vulgaris*-supplemented feed has significantly improved the respiratory burst activity of the fish. The present results are in agreement with the findings of Galal et al. (2018) who also showed that C. *vulgaris* dietary supplementation positively influenced the immune response in tilapia. Prior to that Selim & Reda (2015) showed improvements in the immune response of tilapia when fed with *Bacillus amyloliquefaciens* spores.

Lymphoproliferation activity

Lymphoproliferation, as T-cell and B-cell responses, can also give valuable information on the adaptive immune response (Dupont et al., 2013). Lymphoproliferation activity of the fish spleen cell suspension was measured to assess the enhancement by the *C. vulgaris*-supplemented feed in the procreation of the lymphoid organ cells, as compared to the control group, expressed as stimulation index and spontaneous proliferation of T- and B-cells, respectively.

As shown in Figure 2(Ci), all groups fed with *C. vulgaris*-supplemented feed showed significant enhancement of mitogen-stimulated T-cells proliferation in comparison to the control group, except for group B with the lowest content of *C. vulgaris*. The mitogen-stimulated proliferation was significantly highest in group E, followed by groups C and D which showed no significant difference between their lymphoproliferation activities. Spontaneous T-cells proliferation was also significantly enhanced in all treated groups in comparison to the control group as shown in Figure 2(Cii). The spontaneous proliferation was significantly highest in group E, followed by the rest of the treated groups which showed no significant difference between their lymphoproliferation activities. These results indicated that the *C. vulgaris*-supplemented diet was able to enhance T-immune cells proliferation in tilapia spontaneously, without stimulation with mitogen.

A similar trend was observed for B-cells proliferation as shown in Figure 2(D). The results showed that mitogen-induced proliferation was significantly enhanced in all treated groups in comparison to the control group Figure 2(Di). The mitogen-induced proliferation activity was significantly highest in groups D and E, with no significant difference between their activities themselves. Groups C and B recorded moderate mitogen-induced proliferation activities and were significantly different from each other. Similarly, spontaneous proliferation of B-cells was significantly higher for all treated groups in comparison to the control group as shown in Figure 2Dii. Moreover, there was no significant difference between their proliferation activities. These results indicated that the *C. vulgaris*-supplemented diet was able to enhance B-immune cells proliferation in tilapia spontaneously, without stimulation with mitogen. The results of present study agree with the data obtained which reports the beneficial immunostimulatory effect of short-term *C. vulgaris* supplementation in tilapia feed leading to enhancement of natural killer cell activity and early inflammatory responses (Kwak et al., 2012).

Evaluation of the innate immune responses showed that the spleen immune parameters of the fish exhibited significant enhancements when fed with *C. vulgaris*-supplemented diet. Feed supplemented with 125 mgkg^{-1} bw *C. vulgaris* resulted in

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better improvement in phagocytosis activity while respiratory burst activity was better improved in feed supplemented with 250 mgkg^{-1} bw *C. vulgaris*. Proliferation of immune T-cells was concentration dependent, hence was better improved in fish fed with feed supplemented with 500 mgkg^{-1} bw *C. vulgaris*. On the other hand, proliferation of immune B-cells was better improved in fish fed feed supplemented with 250 to 500 mgkg^{-1} bw *C. vulgaris*. Based on these results, it can be concluded; supplementing fish diet with 125 to 500 mgkg^{-1} bw *C. vulgaris* is effective for immune improvement of tilapia. Moreover, considering that some of the immune parameters were not better improved at the highest concentration of 500 mgkg^{-1} bw *C. vulgaris*, supplementing the feed with as low as 125 mgkg^{-1} bw *C. vulgaris* is already adequate and practical, from the viewpoints of feed digestibility and economics.

^{1}H NMR-based metabolomics

Analysis of the fish spleen samples using ^{1}H NMR-based metabolomics was carried out to understand the metabolic changes related to immune improvement signals affected by feeding with the *C. vulgaris*-supplemented diet. The metabolites present in the spleen samples were identified based on 1D and 2D NMR spectra of treated and control fish samples collected 21 days after the feeding experiment. Figure 3 shows the representative ^{1}H NMR spectra of the spleen extract obtained from groups A to E.

The spectral resonances were assigned by comparing and matching with Chenomx NMR suite (Version 8.3, Alberta, Canada) library, the human metabolome database (HMDB, http://www.hmdb.ca/) (Wishart et al., 2007), and NMR data base (http://www.nmrdb.org/), as well as information from the available literature. Metabolite id was further verified by 2D *J*-resolved and two-dimensional NMR experiments such as HSQC and HMBC. The 2D NMR assignments for some major metabolites involved in the perturbed pathways are presented in the supplementary data (Figure S1). In total, 21 metabolites were identified from the spectral data, presented in Table S4 of the supplementary material.

To understand the metabolite changes in the different fish groups, multivariate data analysis (MVDA) of the spleen NMR spectral data was performed using PLS-DA. In MVDA, the goodness of fit and predictability of a generated model are determined by calculating the R2 and Q2 values, which stands for fitness and predictability, respectively (Verpoorte et al 2007). The R2 value determines how well the training data set can be statistically reproduced. The model is said to exhibit good fitness and predictability and considered valid if the Y-axis intercepts are within R2< 0.3 and Q2< 0.05 (Eriksson et al. 2006). The PLS-DA and PLS models in the present study fulfilled these requirements suggesting that the models met the criteria for the validation and prediction performance. The PLS-DA score plot with PC 1 and PC 2 (Figure 4A), showed a total variance R2Yof 48.3% with a high-class discriminant R2 and Q2 values of 63.1 and 53.0 respectively, indicating that the metabolites were significantly affected by the *C. vulgaris*-supplemented diet. The permutation tests in supplementary data with 100 permutations and external validation indicated that the PLS-DA and PLS models generated during the present study were valid (Figure 6).
There was a clear separation between all the *C. vulgaris*-supplemented diet groups SpB, SpC, SpD, and SpE (supplementation with 62.5, 125, 250, and 500 mgkg^{-1} bw *C. vulgaris,* respectively) and the control group SpA (feed not supplemented with *C. vulgaris*).

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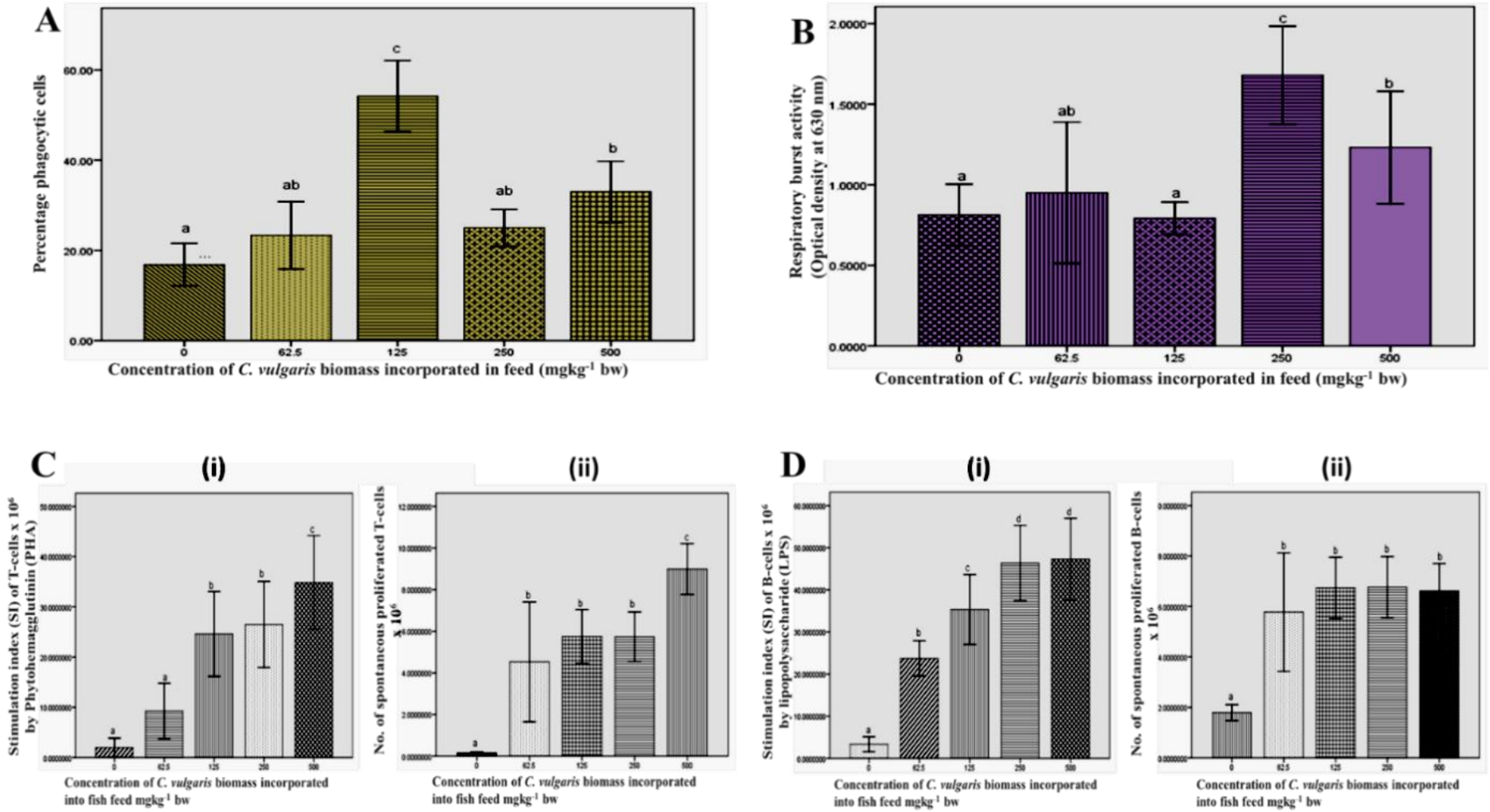

Figure 2: Effect of *Chlorella vulgaris*-supplemented feed on innate immune responses of tilapia: phagocytosis activity (A), respiratory burst activity (B), mitogen-stimulated and spontaneous T-cells proliferation (Ci and Cii), and mitogen-stimulated and spontaneous B-cells proliferation (Di and Dii). Data are expressed as mean ± SE (n=6). Values with the same letters are not significantly different at $p > 0.05$

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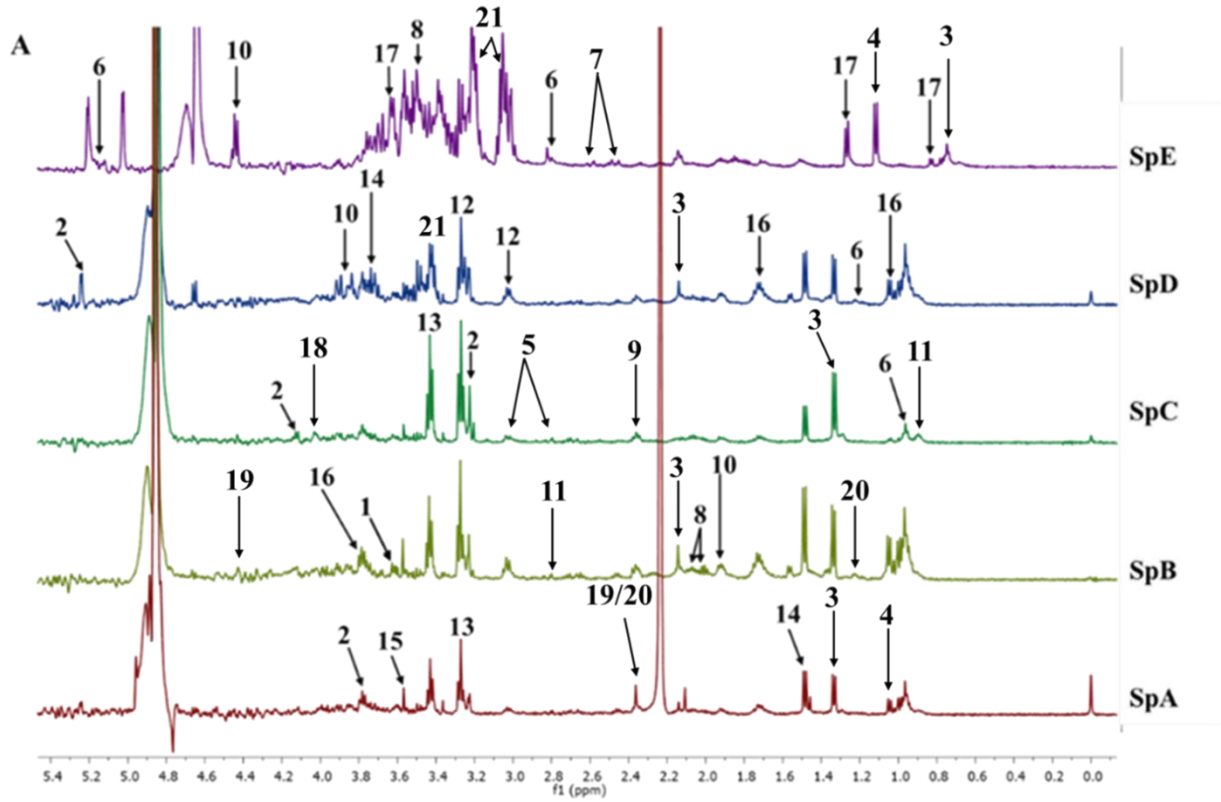

Figure 3: Representative ^{1}H NMR spectra of spleen extract obtained from groups A to E. Choline (1), glucose (2), valine (3), oleic acid (4), creatine (5), linolenic acid (6), methionine (7), glutamic acid (8), palmitic acid (9), proline (10), linoleic acid (11), histamine (12), taurine (13), alanine (14), glycine (15), leucine (16), isoleucine (17), glycolate (18), riboflavin (19), stearic acid (20), arginine (21).

The *C. vulgaris*-supplemented diet groups were clustered together indicating that the groups shared close similarity in their metabolite profiles. The high discrimination of the *C. vulgaris*-supplemented diet groups from the control by PC 1 in the PLS-DA model suggested that their metabolite profiles have experienced significant changes due to the microalgae supplementation. In addition, the spleen samples from SpE, group receiving the highest *C. vulgaris*-supplementation appeared to be slightly separated from other groups by PC2. This could be due to the slighty different metabolite profile of SpE than the lower concentration groups, particularly in the chemical shift range of 3-5 ppm (Figure 3).

Analysis of the PLS-DA loading plot (Figure 4B) revealed that among the significant metabolites contributing to the discrimination were variables identified as taurine, alanine, glucose, glycine and glutamic acid. The significant variables were determined by analyzing the variable importance in a projection (VIP) plot with jack-knifing uncertainty bars, where variables with VIP scores of more than 1 (error bars not crossing the baseline in the loading column plot) were deemed as significant. A total of 11 metabolites met the criteria of being significant metabolites based on their VIP values greater than or equal to 1 (Figure 4D), leading to the identification of the spleen metabolites listed in Table 2 as the important contributing metabolites discriminating the *C. vulgaris*-supplemented diet groups from the control group as visualized by Figure 4A.

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Examination of the PLS biplot (Figure 4C) further showed that the spleen immune parameters (RBA, PHG, and LPA) were correlated in proximity to the *C. vulgaris*-supplemented diet groups, suggesting that the detected metabolites in these diet groups contributed significantly to the immune improvements. The PLS biplot showed that the metabolites alanine, glucose, glycine, and leucine were strongly correlated to RBA, while histamine, taurine, isoleucine, and glucose were strongly correlated to PHG. Additionally, linoleic acid, choline, glucose, leucine, proline, and glutamic acid were strongly correlated to LPA.

Table 2: Metabolites with variable importance of projection (VIP) values ≥ 1.0 in the PLS model of spleen samples

Chemical shift (ppm)	Metabolite	VIP values
δ 2.22	Glutamic acid	9.08
δ 3.26	Histamine	4.23
δ 3.42	Taurine	3.58
δ 3.22	Glucose	2.03
δ 1.46	Alanine	1.70
δ 4.06	Glucose	1.65
δ 3.50	Glycine	1.59
δ 5.02	Linoleic acid	1.35
δ 3.70	Leucine	1.29
δ 4.46	Choline	1.12
δ 3.78	Isoleucine	1.09
δ 4.22	Proline	1.07

The NMR data of the spleen extracts were Pareto-scaled and subjected to Hierarchical Cluster Analysis (HCA) with Euclidean and clustering algorithm using the Ward method (da Silva et al., 2018; Kikuchi et al., 2018; Pontes et al., 2017). A heat map of the differential metabolites found in the above metabolomics analysis is shown in Figure 5. The differential metabolites were represented as squares in the heatmap, their corresponding levels indicated by colors based on a pareto-normalized scale from -1.5 (lowest) to 1.5 (highest).

The heatmap analysis showed that after three weeks of feeding with *C. vulgaris*-supplemented diet, the levels of most metabolites, particularly alanine, taurine, histamine, leucine, glycine linoleic acid, choline, and isoleucine, were higher in the *C. vulgaris*-supplemented diet groups, except for glutamic acid which was higher in the control group. Furthermore, in comparison to the other treated groups, the levels of linoleic acid, choline and glycine were observed to be higher, while glucose and leucine were observed to be lower in SpE. These upregulated levels of alanine, choline, glucose, glycine, histamine, isoleucine, leucine, linoleic acid, proline, and taurine levels in fish fed with *C. vulgaris*-supplemented diet groups compared to the control, are further demonstrated by a box plot of the individual metabolites, presented in supplementary material (Figure S4). The concentration of glutamic acid was negative in the *C. vulgaris*-supplemented diet groups and very high in the control group.

The member compounds from Krebs cycle (TCA cycle) detected in spleen samples which were found to be enhanced as a result of feeding with *C. vulgaris* incorporated diet, especially the amino acids, their abundance is critically involved in inflammatory processes and immune responses of the cells (Alqarni et al., 2019). A fundamental role of taurine in the immune system is protecting tissues from oxidative stress which is associated with the pathology of various inflammatory diseases. The high taurine levels in the *C. vulgaris*-supplemented diet groups are indicative of this important role in the fish innate immunity. Activated phagocytes generate a variety of microbial and toxic oxidants produced by the peroxidase system in these cells. Studies indicated that taurine supplementation in tilapia fish feed could significantly regulate its physiological state and promote growth and development as a result of high level of taurine present in the leucocytes (Shen et al., 2018).

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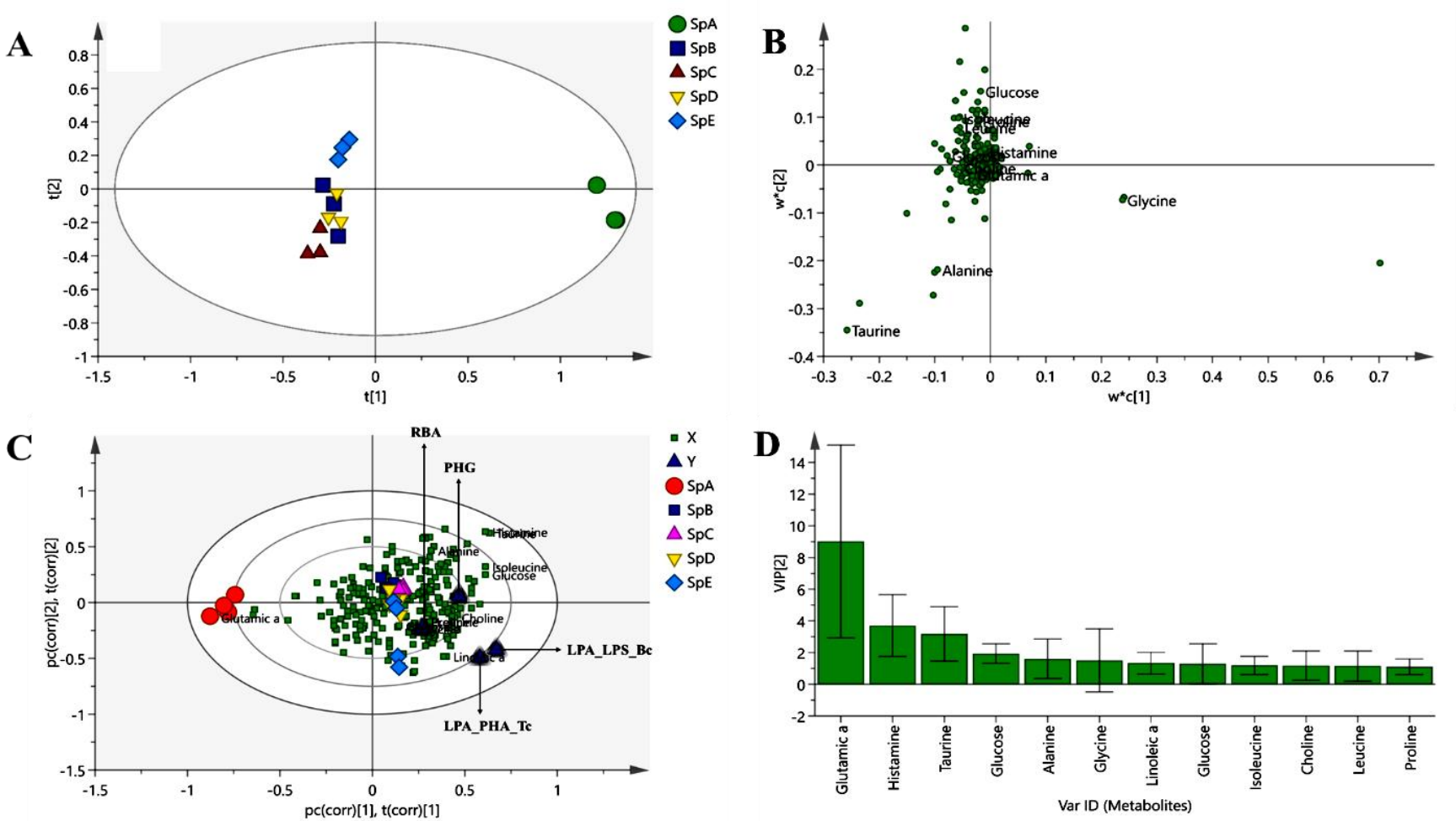

Figure 4: PLS-DA score (A) and loading (**B**) plots generated from ^{1}H NMR spectral data of spleen extracts of control (SpA) and *C. vulgaris*-supplemented diet groups (SpB, SpC, SpD and SpE) (n=6). Loadings in the PLS biplot (**C**) showed the proximity of the identified spleen metabolites to the immune parameters, RBA, PHG and LPA (T-cells and B-cells), while the VIP plot (D) showed the most significant metabolites contributing to the class separation.

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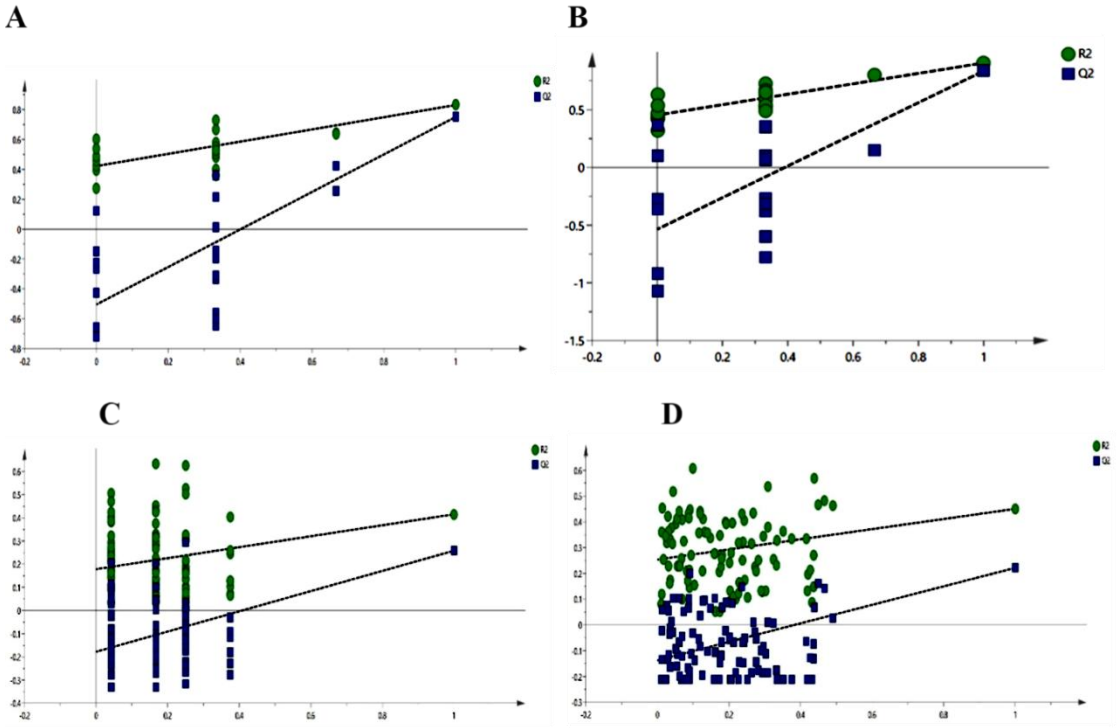

Figure 6: Validation of PLS-DA model for spleen analysis with 100 permutations (A). Validation of PLS model for spleen analysis with 100 permutations for the immune parameters, PHG (B), RBA (C), and LPA (T-cells and B-cells) (D).

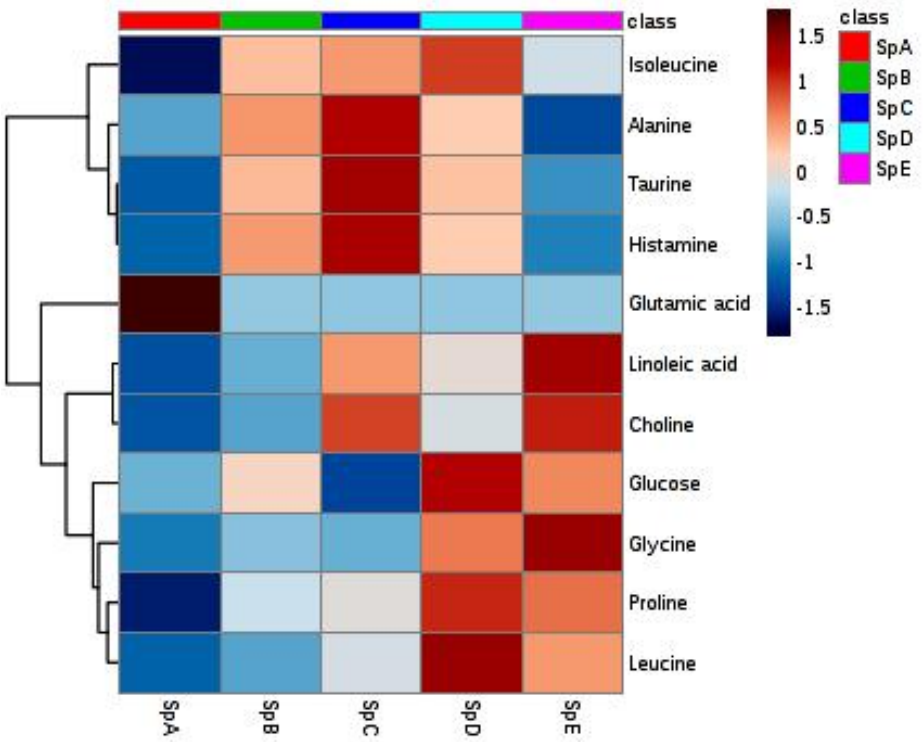

Figure 5: Heatmap showing the varying levels of VIP metabolites in the fish spleen samples.

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Another related studies reported that prolonged taurine deficiency will affect the immune cell functions (Wang et al., 2016). In fact, prolonged taurine deficiency have been reported to lead to profound abnormalities in the immune system including significant leukopenia; a decreased respiratory burst in neutrophils and depletion of B-cells from lymph nodes and spleen (Marcinkiewicz & Kontny, 2014). In addition, it was also reported that upregulation of taurine is responsible for the smooth antioxidant activity, osmoregulation, membrane stability and maintenance of Ca^{2+} stability. Hence, the significant upregulation of taurine levels in the *C. vulgaris*-supplemented diet groups are an indication of an improved level of immunity.

The deficiency of some selected vitamins could affect the induction of proper innate as well as adaptive immune responses. As a result of riboflavin deficiency, typical symptoms called oro-oculo-genital syndrome are observed (Mazur-Bialy et al., 2015). In lymphoproliferation LPS-stimulation model, riboflavin deprived cells were reported to release significantly fewer anti-inflammatory agents. While reduction of the release of proinflammatory factors as well as enhancement of anti-inflammatory mediators were both recorded with riboflavin enriched model (Mazur-Bialy et al., 2015). It was observed in this study that the level of riboflavin was enhanced in the treated groups as compared to the control group indicating improved immunity level in treated groups. Histamine is an inflammatory mediator and impact the immune system, usually as proinflammatory factors. It acts as a regulatory component to establish homeostasis after injury or prevent the inflammatory process (Branco et al., 2018). Histamine is an important chemical mediator that causes vasodilation and increased vascular permeability and may even contribute to anaphylactic reactions. It also acts on several physiological functions, such as cell differentiation, proliferation, haematopoiesis, and cell regeneration (Owens et al., 2011). The sharp increase of histamine in treated groups in this research is an indication of improved immunity level by the incorporation of *C. vulgaris* in fish feed. Glucose is essential for innate immune cells to mount effective anti-fungal responses (Tucey et al., 2018). Tucey et al., 2018 show that, for infected macrophages, dependence on glucose becomes their downfall: fungal pathogen *Candida albicans* rapidly consumes glucose, causing macrophages to die. Likewise, maintaining host glucose homeostasis is important to prevent life-threatening fungal infection (Tucey et al., 2018). It is well known that excess glucose is also a non-healthy metabolic profile. Therefore, the substantial up-regulation in glucose level in the treated fish population resulting from this research, indicate swift innate immune improvement because of *C. vulgaris* incorporation into fish diet.

An increase in the level of choline in the treated groups is a possible indication of mediation of different immune pathways. It's up-regulation could be due to an increase demand for immune-regulatory of some signalling molecules with immunomodulatory effects as a result of their tendency to activate related nuclear receptors (Gray et al., 2015). Intra cellular levels of many amino acids were reported to sharply increase, indicative of increased biosynthesis required for T-cells growth and proliferation (Everts, 2018). In addition, intracellular pools of fatty acids are elevated, presumably acting as a pool to generate cell membranes suitable for immune cells proliferation (Everts, 2018). Thus, the levels of alanine, proline, glycine, choline, linoleic acid, stearic acid, and linolenic acid in this research, were noticed to either sharply increased or just relatively enhanced in the treated groups as compared to the control fish population.

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Glutamic acid is a critical fuel source for immune cells, including white blood cells (De Oliveira et al., 2016), however, its levels can be decreased due to major injuries that led to excessive blood loss (Lin et al., 2013; Van Zanten et al., 2015). If a body's need for glutamic acid is greater than its ability to produce it, the body may break down protein stores such as organ muscles, to release more of this amino acid (Bettina et al., 1999). In this research, all available blood from the caudal vein of fish samples were initially drained before the fish was sacrificed and its spleen removed. For these reasons, it may explain why the level of glutamic acid was detected to be low in the spleen extracts

Metabolite Association and Pathway Analysis

To identify novel pathways for the improved immunogenesis, untargeted metabolomics is an applicable technique. The driven insight and biomarkers identified by this approach can be used to extract the desired information about immunogenesis triggered by immune-stimulants and the related metabolites regulations. The Metabolomics Pathway Analysis (MetPA) coupled with Kyoto Encyclopedia of Genes and Genomes (KEGG) can be a useful approach for such pathway analysis. This combined methods revealed 9 metabolites with high hits in the 21 pathways (Figure 7).

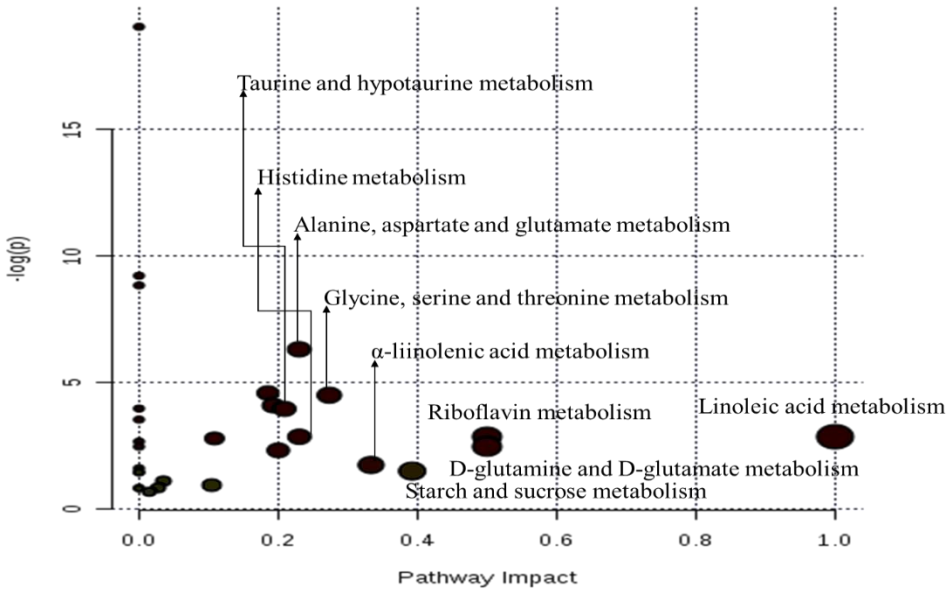

Figure 7: Pathway analysis show the various pathway impact factors.

Linoleic acid metabolism; riboflavin metabolism; D-glutamine and D-glutamate metabolism; starch and sucrose metabolism; α-linolenic acid metabolism; glycine, serine and methionine metabolism; alanine, aspartate and glutamate metabolism; histidine metabolism; taurine and hypo taurine metabolism possessed the highest hits, with values of 1, 1, 1, 1, 1, 3, 2, 2 and 1 respectively. Linoleic acid metabolism had the highest pathway impact factor, followed by riboflavin metabolism, D-glutamine and D-glutamate metabolism and starch and sucrose metabolism, with impact factors of 1.00, 0.50, 0.50 and 0.39, respectively. The pathway impact factor was calculated by considering the matched metabolite's importance in the network. In addition, a detailed pathway analysis was designed and proposed to show the possible

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association among the metabolic pathways of the most significantly affected metabolites (Figure 8). There were eight important metabolites significantly upregulated in the *C. vulgaris*-supplemented groups: taurine, choline, linoleic acid, glucose, glycine, proline, histamine, and alanine that are involved in some metabolic pathways suggested by the Kyoto Encyclopedia of Genes and Genomes (KEGG).

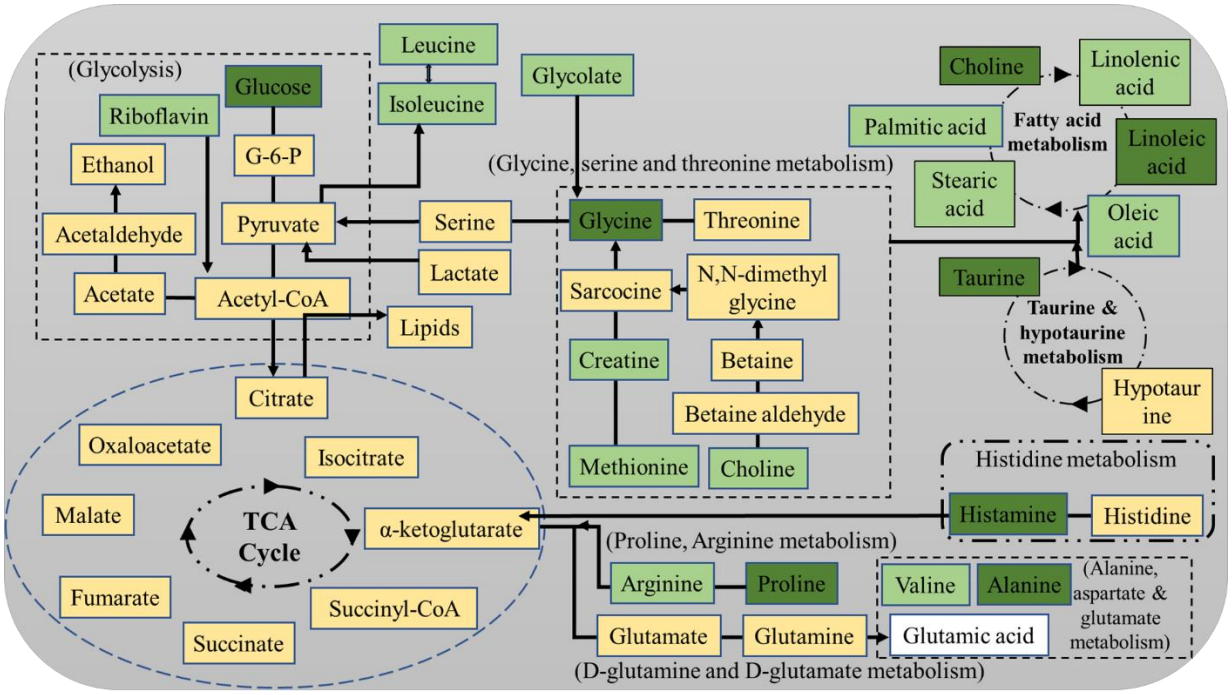

Figure 8: Proposed metabolic pathways affected by *C. vulgaris*-supplemented diet as detected in the spleen of tilapia: metabolites in dark-green and white boxes represent up-regulated and down-regulated levels, respectively, in *C. vulgaris*-supplemented groups compared with the control group. Metabolites in green and yellow boxes respectively represent those detected and not detected during analysis.

Considering perturbation in metabolism; The experimental results demonstrate that the upregulation in the level of taurine caused significant dietary-dependent and time/development-dependent metabolic regulations in tilapia, particularly regarding the TCA cycle and glycolysis/gluconeogenesis, amino acid metabolism, lipid metabolism, and fatty acid metabolism (Safwan et al., 2022) (Figure 8). According to a previous study, taurine supplementation could act in synergy with insulin or insulin-like substances to further promote the usefulness of amino acid and glucose uptake in cells. Consequently, taurine supplementation would decrease the levels of some amino acids while increasing others to accelerate the glycolysis/gluconeogenesis, promoting the fish body growth (Shen et al., 2018). This indicates that taurine supplementation would promote rapid growth of fish in the early and middle growth stages (Dad & Ikhwanuddin, 2012).

The level of glucose in the present study were shown to be upregulated, the increase could be due to the increased glycolysis which occurred in order to meet the energy demand for fish growth and development (Shen et al., 2018). This might be due to the need to produce energy in biological systems which is highly correlated to glucose metabolism. This process involves the

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catabolizing of glucose through glycolysis into pyruvate, which might be transformed to acetyl-CoA with the aid of riboflavin. Consequently, the synthesis and utilization of ketone bodies are elevated due to glucose upregulation (Mediani et al., 2018). The tricarboxylic acid (TCA) cycle is the process that converts acetyl-CoA into energy in the presence of carbon dioxide in the mitochondria. These processes take place via the oxidation of the acetate derived from proteins, carbohydrates, and fats metabolism (Mediani et al., 2018).

The amino acids upregulated in the *C. vulgaris*-supplemented groups; taurine, glycine, proline, alanine and their derivative histamine, their upregulation is an indicative of an improved inflammatory processes and immune responses of the lymphoid cells (Alqarni et al., 2019).

CONCLUSIONS

The present study describes for the first time the application of metabolic approach in analysing tilapia spleen cells which was administered *C. vulgaris*-supplemented diet. Results of our findings revealed that *C. vulgaris*-supplemented diet at a concentration of 125 $mgkg^{-1}$ bw resulted in better improvement in phagocytosis activity while respiratory burst activity was better improved in feed supplemented with 250 $mgkg^{-1}$ bw *C. vulgaris*. Proliferation of immune T-cells was concentration dependent, hence was better improved in fish fed with feed supplemented with 500 $mgkg^{-1}$ bw *C. vulgaris*. On the other hand, proliferation of immune B-cells was better improved in fish fed feed supplemented with 250 to 500 $mgkg^{-1}$ bw *C. vulgaris*. Based on these results, it can be concluded that; supplementing fish diet with 125 to 500 $mgkg^{-1}$ bw *C. vulgaris* is effective for immune improvement of tilapia. Moreover, considering that some of the immune parameters were not better improved at the highest concentration of 500 $mgkg^{-1}$ bw *C. vulgaris*, supplementing the feed with as low as 125 $mgkg^{-1}$ bw *C. vulgaris* is already adequate and practical, from the viewpoints of feed digestibility and economics. The PLS biplot showed that the spleen immune parameters (RBA, PHG, and LPA) are correlated in proximity to the treated groups, suggesting that detected metabolites from the treated groups contributed significantly to the immune improvements. Furthermore, the PLS biplot showed that the metabolites alanine, glucose, glycine, and leucine were strongly correlated to RBA, while histamine, taurine, isoleucine, and glucose were strongly correlated to PHG. Additionally, linoleic acid, choline, glucose, leucine, proline, and glutamic acid were strongly correlated to LPA. It was also observed that all the mentioned metabolites except for glutamic acid, were up regulated in fish spleen because of administered *C. vulgaris*-supplemented diet.

Results from MVA reveals that ^{1}H NMR metabolomics is potential analytical tool for the identification of biomarkers in *C. vulgaris* induced immune stimulation via its incorporation in fish diet in minimum concentration and time of treatment. To the best of our knowledge, the present study was the first attempt that proposed the biomarkers along with potential pathways associated with immune enhancement in tilapia fish by *C. vulgaris* microalgae incorporation into fish diet which paves a way for future development in mitigating disease outbreak for the development of aquaculture practice. However, further research is required to explore the application of *C. vulgaris* to tilapia during microbial infection before concluding on its application as an alternative to the available classical modalities used in managing tilapia fish ailments.

Supporting Information: Table S1: Upscale parameters for culture medium of C. vulgaris sample procurement, Table S2: Proximate analysis of the re-pelleted commercial fish feed supplemented with different

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concentrations of *C. vulgaris*, Table S3: Serum biochemical analysis, Table S4: Representative ^{1}H NMR detected metabolites in spleen samples showing the chemical shifts, Figure S1: 2D NMR spectral elucidation of some major VIP metabolites responsible for the discrimination between varied observations and which were involved in the perturbed pathways with high impact factors, Figure S2: Histological profile of fish organs, Figure S3: Phagocytic and non-phagocytic cells, Figure S4: The box plot of detected VIP metabolites in spleen of fish samples.

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