EFFECTS OF ALGAE FEEDING ON MOUSE METABOLOME

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ABSTRACT

Algae have been investigated and developed as a source of food, dietary supplement, and biofuel, due to their chemical and nutrient composition. Algae consumption carries algal proteins, polyunsaturated fatty acids (PUFAs), vitamins, dietary fibers, and bioactive compounds into the biological systems of humans and animals, and therefore are expected to elicit metabolic and physiological responses. Numerous efforts have been undertaken to understand the health-promoting effects of algae consumption, such as their hypolipidemic, antioxidant, anti-obesity and anti-cancer properties. However, the metabolic events in algae-elicited effects were not examined in details in spite of the fact that these benefits are largely based on the metabolic interactions between algal components and the biological system. In this study, the influences of consuming green algae (*Scenedesmus sp.*) on the metabolic status of young mice was investigated through growth performance, blood chemistry, and liquid chromatography-mass spectrometry (LC-MS)-based metabolomics. Compared to the control diet, 5% algae promoted growth performance while 20% algae suppressed it. The growth performance was significantly increased by 5% algae but decreased by 20% algae feeding. Serum glucose, triacylglycerols (TAG), and blood urea nitrogen (BUN) levels were not affected by both treatments, but serum cholesterol level was dramatically decreased by 20% algae feeding. Metabolomic analysis of liver, serum, feces and urine samples revealed diverse influences of algae feeding on mouse metabolome, which are represented by the features as follows: 1). Urinary vitamins and fecal pigments are identified as robust exposure markers of algae feeding. 2). Despite the high-level protein in algae, the impacts of algae

feeding on free amino acids in serum and the liver were quite limited. 3). Algae feeding increased the PUFA levels in serum and liver lipidomes and the free fatty acids in feces. 4). 5% algae increased the level of reduced glutathione (GSH) in the liver while 20% algae increased the level of oxidized glutathione (GSSG) in the liver and the levels of aldehydic lipid oxidation products (LOPs) in the liver and urine. 5). 5% algae selective increased the levels of intermediate metabolites, including adenosine monophosphate (AMP), adenylsuccinic acid, dephospho-CoA, and nicotinamide, in the liver while 20% algae increased the levels of carnitine and carnitine derivatives in the liver. 6). Algae feeding dramatically altered the microbial metabolism, as reflected by the increases in short-chain fatty acids (SCFAs) and primary bile salts in feces, the increases of branched fatty acids in urine, the decreases of secondary bile acids in feces, and the decrease of p-cresol metabolites in urine. Overall, multiple correlations between metabolite markers and growth performance in algae feeding were established in this study and could serve as a foundation for further mechanistic investigations on the biological effects of algae feeding.

TABLE OF CONTENTS

List of Tables

List of Figures

CHAPTER 1 LITERATURE REVIEW

[1]**KEY WORDS:** algae, nutrients, bioactive compound, metabolomics

ABBREVIATIONS: 4-HNE, 4-hydroxynonenal; AIDS, acquired immune deficiency syndrome; APCI, atmospheric pressure chemical ionization; CO_2, carbon dioxide; CVD, cardiovascular disease; DGDG, digalactosyldiacylglycerol; DHA, docosahexaenoic acid; DNA, deoxyribonucleic acid; EPA, eicosapentaenoic acid; ESI, electrospray ionization; FAD, flavin adenine dinucleotide; FAO, the food and agriculture organization; FFA, free fatty acid; FMN, flavin mononucleotide; FT, Fourier transform; GC, gas chromatography; GC-MS, gas chromatography mass spectrometry; HDL, high-density lipoprotein; HPLC, high performance liquid chromatography; LC, liquid chromatography; LC-MS, liquid chromatography mass spectrometry; LDL, low-density lipoprorein; MDA, malondialdehyde; MDA, multivariate data analysis; MGDG, monogalactosyldiacylglycerol; MS, mass spectrometry; MUFA, monounsaturated fatty acid; NMR, nuclear magnetic resonance spectroscopy; OPLS, orthogonal partial least squares; PA, phoshatidic acid; PAG, protein-calorie advisory group; PC, principle component; PC, phosphatidylcholine; PCA, principal component analysis; PE, phosphatidylserine; PG, phosphatidylglycerol; PLS-DA, partial least squares-discriminant analysis; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; RT, retention time; TAG, triacylglycerol; UCP1, uncoupling protein 1; UPLC, ultra-pressure liquid chromatography.

1.1 INTRODUCTION TO ALGAE

1.1.1 Overview of algae

Algae are ancient species. The term ‘algae’ refers to a large and diverse group of polyphyletic and mostly photosynthetic organisms, which have different origins, evolutionary lines and biochemistry (1). In contrast to plants, algae do not have true roots, stems, leaves, vascular tissue and have simple reproductive structures. At present, over 100,000 species have been identified (2). In addition, they vary in forms and sizes as some marine seaweed can exceed 50 meters in length. Based on the size, algae are classified as microalgae and macroalgae. Classification of algae is summarized in **Figure 1.1**.

Macroalgae, also called seaweed, are multicellular organisms growing in salt or fresh water. Based on specific pigments, they can be classified into three broad divisions: brown algae (Phaeophyceae), red algae (Rhodophyceae) and green algae (Chlorophyceae) (3,4). Macroalgae are mainly utilized for the production of food and the extraction of hydrocolloids (5).

Microalgae constitute a highly diverse group of prokaryotic and eukaryotic organisms. As microscopic organisms, they are typically found in benthic or littoral zones of water (1). Microalgae have the photosynthetic mechanism similar to land-based plants. Being surrounded in an aqueous environment with access to sunlight, water, CO_2, and other nutrients, microalgae are highly efficient in converting solar energy into biomass. The classification of microalgae is based on the properties such as pigmentation, chemical

nature of photosynthetic storage product, the organization of photosynthetic membranes, and other morphological features (5). The most abundant microalgal classes are Cyanophyceae (blue-green algae), Chlorophyceae (green algae), Bacillariophyceae (including the diatoms), and Chrysophyceae (including golden algae) (6). Within these classes, microalgae belonging to *Spirulina, Chlorella,* and *Dunaliella* genera have been widely consumed by humans and used in commercial production (7). For example, *Spirulina platensis*, a free-floating filamentous species preferably growing in alkaline water, contains higher amounts of protein, vitamins and minerals than many other microalgae. Its applications as dietary supplement include weight loss, lowering cholesterol, and antioxidant (8). *Chlorella vulgaris* is also used as a dietary supplement, a food additive, and a dye. It has the highest level of chlorophyll in all edible species, and is also a good source of polyunsaturated fatty acids and vitamin B12. *Dunaliella salina* is enriched with carotenoids, such as β-carotene, a precursor of vitamin A (9). It had been used to support skin and eye health, and to maintain a healthy immune system (10). Besides these three microalgae species, other microalgae have also been explored for the applications in food industry, environmental treatment and health promotion. For example, *Scenedesmus* species decreased the cholesterol level in the animal feeding experiment (11), and are also widely used in wastewater management to remove excessive nitrogen and phosphorus (12).

1.1.2 Historical and current use of algae

The concerns on the possibility that the population growth would outstrips the food production in the world in 1890s and 1940s resulted in the exploration of alternative sources of food for human consumption. Algae were recognized as a potential solution for global hunger due to their extraordinary productivity (13). In 1948, *Chlorella* was found to be able to convert about 20% of available solar energy into the biomass that contains 50% protein when dried, but mass production of *Chlorella* was hampered by the requirements of specific temperature, density, light, pH and nutrients (14). At the same time, global production of grains and soybean surged, making algae production less economically viable. After ending the efforts of producing algae to solve hunger, the efforts of producing algae for American astronauts did not stop until the contamination became an unsolved problem (15). The remaining efforts on the human consumption of algae in late 20^{th} century were mainly dedicated to the development of algae as commercial food supplements.

Algae have been considered as a fuel source for long time. Production of methane gas from algae was proposed in early 1950s, and accepted as an incentive during the energy crisis in 1970s. In the past three decades, significant research and development efforts have been dedicated to improve the efficiency of producing oil from algae.

Dietary supplement and biofuel are not the only applications of algae. Utilization of algae in waste water treatment is another important application. The research on cultivation of algae in waste streams dated back to early 1950s. Algae offer many advantages over traditional treatment on waste water, including reducing toxic chemicals and heavy

metals from waste water and recovering the carbon and nitrogen contents for nutrient production (**Figure 1.2**).

1.2 CHEMICAL COMPOSITION OF ALGAE

Genes and environment determine the chemical composition and nutrient content of algae. The influences of genetic background are reflected by the fact that chemical composition of algae differs from strain to strain, while environment parameters such as temperature, pH value, illumination, and mineral content greatly affect the metabolic activities of algae (**Table 1.1**) (16). However, the levels of major biochemical fractions such as protein, lipid and carbohydrate may be unrelated to the nutritional quality of algae. Instead, the levels of specific amino acids, fatty acids, sugars, vitamins and minerals may be more important (17). Therefore, a desirable composition of chemicals and nutrients in algae could be achieved through the species selection and the modification of culture conditions and physical parameters (18).

1.2.1 Protein and amino acids

Protein content is an important factor determining the nutritional value of algae. High protein content has been observed in various algae, making them unconventional sources of protein (19). Reported concentrations of algal proteins, dominantly enzymatic proteins, were mostly determined using the crude protein measurement method, which is

commonly used in the evaluation of food and feeds (20). The method determines the total nitrogen level after hydrolyzing algal biomass. Because nucleic acid, amines, glucosamides, and cell wall materials also contain nitrogen, this method could overestimate the true protein content in algae (16).

Amino acid composition of marine and freshwater algae has been examined in multiple studies (17,21). The results suggested the levels of essential amino acids in algae are relatively high than other foods. However, one exception is lysine, which has a lower proportion in most algae (22,23). Moreover, the levels of methionine and cysteine are low or absent in most algae species (**Table 1.2)** (24).

1.2.2 Carbohydrates

Algae are photosynthetic organisms, which mean that carbohydrates, including monosaccharides, disaccharides, and polysaccharides, are formed either as the intermediates or end products of their metabolic processes. Carbohydrates serve two main purposes in algae. One is acting as the structural component in the cell walls, such as fibers, while the other as storage components inside the cell. The stored carbohydrates are species-dependent. Cyanobacteria synthesize glycogen, red algae synthesize floridean starch and green algae synthesize amylopectin-like polysaccharides (25,26).

1.2.3 Lipids

Lipids are responsible for energy storage, membrane barriers, and signaling. Lipid content in algae varies from 1% to 40% of the dry weight. However, under certain conditions, it can go as high as 85% of the dry weight (27). Algal lipids consist of non-polar glycerolipids (neutral lipids), phospholipids, glycolipids, and free fatty acids (FFA). Triacylglycerols (TAG) are the most prevalent neutral lipid found in algae, acting as a storage product and energy reservoir. The major phospholipids in algae are phosphatidylglycerols (PG), phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylserines (PS), and phoshatidic acids (PA). Typical algal glycolipids include monogalactosyldiacylglycerols (MGDG), digalactosyldiacylglycerols (DGDG) and sulfolipids (28).

Most of the naturally fatty acids found in algae have even carbon number, either saturated or unsaturated, range from C12 to C22, but odd chain FAs also exist. Based on the numbers of double bonds present, those FAs are classified as monounsaturated FAs (MUFAs) and polyunsaturated FAs (PUFAs). Further, PUFAs can be categorized into n-3 PUFAs and n-6 PUFAs depending on the position of the first double bond from the methyl end (29,30). Algal n-3 PUFAs have unique nutritional and pharmacological values since they are not synthesized by humans and animals and have to be obtained from the diet. Among n-3 PUFAs, α-linolenic acid is regarded as a bioactive component for lowering high blood pressure and plasma cholesterol level, and can be further converted into docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Beneficial effects of DHA and EPA on diabetes mellitus, hypertension and neurogenesis have been observed (31). Within algal n-6 PUFAs, arachidonic acid can exert pharmacological effects through their metabolites, including prostaglandins, prostacyclins and leukotrienes

(32,33). For example, *Scenedesmus* contains palmitic acid (C16:0) as the most abundant saturated fatty acid, while oleic acid (C18:1), linoleic acid (C18:2), and eicosapentaenoic acid (20:5) are the most abundant unsaturated fatty acids (**Table 1.3**) (34,35).

1.2.4 Nucleic acids

Nucleic acids are basically present in all living organisms, including algae. However, they are considered as toxin under certain circumstances. For humans, during the digestion, nucleic acids produce uric acid, which can precipitate and form sodium urate crystal deposits in cartilage, resulting in gout. Also, the elevated level of uric acid in urinary levels may increase the risk of forming uric acid stones in the kidney and nephropathy (36). The average nucleic acid content in algae varies between 4-6% of the dry matter, compare to the 8-12% in yeast and up to 20% for bacteria. Thus, the high content nucleic acid content in algae limits its use as food (37). According to the United Nations Protein-Calorie Advisory Group (PAG), the recommendation for the daily consumption of nucleic acid from unconventional sources should not exceed 2 g, with total nucleic acids from all sources not exceeding 4 g per day, which approximately equals about 40 g of algae per day (27).

1.2.5 Vitamins

The enrichment of vitamins in algae is another factor behind their usage as dietary supplements in humans. Vitamins are vital organic nutrients that are needed by living organisms in limited amounts. algae contain both water-soluble vitamins (B1, B2, B12, C) and fat-soluble vitamins (A, E, K) (38). The concentration of vitamin in algae is affected mainly by species, algal growth stage, geographic area, availability of light and temperature (**Table 1.4**).

Vitamin B_2: Riboflavin is the central component of the flavin cofactors of metabolizing enzymes, mainly flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). As electron carrier, these flavin cofactors participate in the redox reactions responsible for energy transfer and redox balance (39). Because of its water solubility, riboflavin is continuously excreted in the urine, making the deficiency more common. The average level of riboflavin in algae is between 40 to 60 mg/kg of dry matter, while beef liver is 29 mg/kg (40).

Vitamin B_{12}: Many B vitamins exist in both algal species and plants. The exception is vitamin B_{12} or cobalamin, which is not synthesized by plants, but abundant in algae (1). Vitamin B_{12} is a cofactor for the methylation reactions in the synthesis of nucleic acids and neurotransmitters, and therefore plays an important role in the development and functioning of brain and nervous system (41). *Spirulina* and *Chlorella* are enriched with vitamin B_{12}. Daily consumption of one gram *Spirulina* is supposed to meet the daily

requirements in B_{12} for humans (42,43). However, controversies also exist on the role of algae as the providers of vitamin B_{12} since the cobamide in algae is considered as pseudovitamin B_{12} is with limited bioactivities and bioavailability (41,44). At present, algae are not recommended as a reliable source of vitamin B_{12} for vegetarians. Further studies are required to define the biological functions of vitamin B_{12} from algae source (45).

Vitamin A: Vitamin A is essential for vision, bone growth, reproduction and immune system health. Animal source of vitamin A is called retinol, which is bioavailable and can be used by body directly. Algae contain provitamin A carotenoids, instead of vitamin A. Beta-carotene is the most abundant carotenoids in algae. For example, β-carotene accounts for 50% of carotenoids in *Spirulina* and *Dunaliella*. Once absorbed, β-carotene can be converted to retinal by a β-carotene-15,15'-dioxygenase-mediated reaction (46).

Vitamin C: Vitamin C is important antioxidant in humans and animals. It can act as electron donor for enzymes and can strengthen the immune defense system.. In general, algae contain high level of vitamin C (1). The average level of vitamin C concentration is 500 to 3000 mg/kg of dry matter for the green and brown algae, whereas the red algae have a lower concentration around 100 to 800 mg/kg (47).

1.2.6 Minerals

Minerals are essential for optimal health through their functions as energy gradient builder, regulatory cofactors, and signaling molecules. Many essential minerals and trace elements are present in marine algae (48). Their levels in algae are commonly higher than of the levels in land plants and animal products. Therefore, the edible marine algae may be an important source of minerals for human since some of the trace elements are rare in land vegetables (49). The levels of various mineral contents usually depend on algae genera, geographical origin, and environmental variations (50). For example, many marine algae are enriched with iodine and iron, making them excellent food supplements to improve dietary intake of iodine and iron (51). Furthermore, *Spirulina platensis* is rich in potassium, *Chlorella vulgaris* is a good source of phosphorus, and *Isochrisis galbana* contains high levels of calcium, magnesium, and selenium (52).

Heavy metals: Many algae are efficient in absorbing metals from the environment. It has been demonstrated that algal cells can be saturated by heavy metals within 24 h (27). This property led to the application of algae in heavy metal bioremediation (53,54). However, on the other hand, heavy metal contamination becomes a concerning issue when algae are consumed by humans and animals, or its heavy metal content moves through the food chain. The levels of heavy metals, including lead and copper, in algae are largely determined by the environment, and therefore can vary greatly. At present, no U.S. government standards exist for the levels of heavy metals in algal products. WHO/FAO guidelines suggest that an adult person of 60 kg body weight should not incorporate more than 3 mg of lead, 0.5 mg of cadmium, 20 mg of arsenic, and 0.3 mg of mercury per week through diets.

1.2.7 Pigments

The classification of algae as brown algae, red algae, and green algae is based on their colors, therefore the pigments in algae (55). Chlorophylls, carotenoids and phycobiliproteins are three major groups of pigments found in algae. Each group of these pigments has distinctive bioactivities and nutritional values (56).

Chlorophylls: Chlorophylls, existing as green lipid soluble pigments, are vital for photosynthesis. Chlorophylls have different forms (**Figure 1.3A**). Chlorophyll-a is the key photosynthetic pigment in all algae and it is the only chlorophyll can be found in cyanobacteria and the Rhodophyta (27). Green algae and land plants also contain chlorophyll-b. Additional forms of chlorophyll –c, -d, and –e can be found in red algae, brown algae, and dinoflagellates (57).The total amount of chlorophyll in algae is around 0.5-1.5% of dry weight. The nutritional values of chlorophylls in algae is controversial. Chlorophylls can be converted into pheophytin, pyropheophytin and pheophorbide in food processing. These derivatives possess antimutagenic activity and are implicated as potential cancer preventive agents. On the other hand, the degradation products of chlorophyll have been identified as a cause of skin irritations (27).

Carotenoids: Carotenoids is a class of mainly yellow, orange, or red fat-soluble pigments with aliphatic or alicyclic structure, which give the color to plant parts and act as accessory pigments for light-harvesting process during photosynthesis (1). There are more than 750 structurally defined carotenoids found from nature including land plants,

algae, and bacteria, of which are comprised of 40 branched carbon units bonded together (58). Two major classes of carotenoids were classified: xanthophylls (which contain oxygen) and carotenes (which are composed of oxygen-free hydrocarbons). Lycopene is the precursor of all carotenoids found in algae, which is synthesized by stepwise desaturation of the first 40-carbon polyene phytoene (27). Many different kinds of caroteinoids were found in algae, with certain like β-carotene, fuxocanthin and astaxanthin show in most algal classes (**Figure 1.3B**), while others are found only in some algal divisions and therefore can be used as chemotaxonomic markers (59,60).

In general, green algae contain β-carotene, lutein, violaxanthin, neoxanthin and zeaxanthin, while red algae mainly contain α- and β-carotene, lutein and zeaxanthin. Brown algae were reported containing β-carotene, violaxanthin and fucoxanthin (61). The average concentration of carotenoids in most algae is only 0.1-2% of the dry weight, but under certain conditions with high salinity and light intensity, *Dunaliella* can produce up to 14% of β-carotene of the dry weight, as reported (27).

It has been proved that β-carotene isolated from algae can be used as food additive if it was of sufficient purity to meet the specifications for synthetic β-carotene (62). β-Carotene plays an important role in food industry, acting as a food coloring or improving the health and fertility of grain fed cattle. Also, it is the primary dietary source of provitamin A and has some nutrition value on cancer prevention due to the antioxidant properties. Nowadays, *Dunaliella salina* and *D. bardawil* have been exploited commercially due to their high accumulation of β-carotene (27).

Phycobiliproteins: Unlike chlorophylls and carotenoids, phycobiliproteins, a complex between proteins and covalently bound pycobilins, are water-soluble proteins present in cyanobacteria and certain algae (1). The function of phycobiliproteins is to capture light energy and pass it to chlorophylls in photosynthesis. Phycoerythrin and phycocyanin are two major types of phycobiliproteins (**Figure 1.3C**) (63). Phycoerythrin is a red pigment, usually present in red algae and cryptophytes, whereas phycocyanin is a blue pigment, which can be isolated from red algae and cyanobacteria (64). Phycobiliproteins, especially phycoerythrin, can account for about 1.2% of dry weight in red algae as a major proportion of cell proteins. Phycobiliproteins are used in chewing gum and dairy productions as natural colorants. Further, algal phycobiliproteins have been shown antioxidant and anti-inflammatory activities (65).

1.2.8 Algal toxins

In some region of South America, Europe, Asia, South Africa and Australia, poisoning man or livestock by algal toxins are not frequency, but unpredictable. Although algal blooms historically have been considered as a natural phenomenon, the agriculture runoff and other pollutants of freshwater and marine have resulted increased nutrient loading of phosphorus and nitrogen, which providing conditions favorable to the growth of toxic algae (66). Toxic algal strains are predominantly cyanobacteria, such as *M. aeruginosa*, *Anabaena flos-aquae* and *Aphanizomenon flos-aquae*, which are often morphologically indistinguishable from non-toxic strains (27). Others algal toxins including domoic acid, saxitoxin, and brevetoxin have been suspected, but rarely been documented (**Table 1.5**).

They poisoned marine life, from shellfish to sea lion, ending up in human food chain through the consumption of seafood products (67). Although no cases were reported in connection to mass-cultured algae, the detailed investigations for the presence of toxins, including chemical analysis, toxicity studies with animals, as well as investigations on antibiotic activities should be addressed in the future.

1.3 BIOLOGICAL PROPERTIES OF ALGAE AND ALGAL COMPONENTS

Biological function of a compound is directly associated with its structure. Diverse structures of nutrients and phytochemicals in algae implicate diverse biological functions. For example, the consumption of algae and algal products as functional food is mainly based on the potential health benefits of algal components, such as omega-3 fatty acids and pigments (6). However, negative impacts of algal components on wellbeing and homeostasis have also been noticed in feeding studies (27,68).

1.3.1 Health promoting effects of algae

1.3.1.1 Antioxidant properties of algae and algal components

Oxidative stress is caused by the imbalance between the production of reactive oxygen species (ROS) and the protective functions of antioxidant system (69). It is a driving force behind many adverse health events, contributing to the pathogenesis of atherosclerosis, diabetes, neurodegenerative diseases, AIDS, chemical carcinogenesis,

and aging (69-72). Free radicals are the most important ROS. Because free radical contain one or more unpaired electrons, they can readily react with DNA, proteins, and lipids, disrupting their normal functions and inducing stress signals or mutations (73,74).

Many algae contain significant amounts of antioxidants (detailed in 1.2.5 and 1.2.7). Among all pigments found in algae, β-carotene, one of carotenoids, plays an important role in the antioxidant activity of lgae since it can inhibit singlet oxygen-mediated lipid peroxidation (75). Astaxanthin, a keto-carotenoid, is another free radical scavenger with the activity 500 times higher than an vitamin E (76). Besides carotenoids, phenolic compounds are also natural antioxidants. (77).

The antioxidant activities of algae have observed in both *in vitro* and *in vivo* studies (78-80). For example, after observing the decrease of the production of oxidative markers such as malondialdehyde (MDA) rat brain homogenate by the extract of *Spirulina* in an early study (81), the protective effects of *Spirulina* against spontaneous lipid peroxidation were confirmed by the decrease of 4-hydroxy-2-nonenal (4-HNE) and the increase of reduced glutathione (78,82) A comparison on the antioxidant activity of 23 species of algae found out that *Scenedesmus obliquus* possess a particularly high antioxidant activity, suggesting its potential usage as a good choice of antioxidant additives for food formulation in the future (**Figure 1.4**) (83).

Compared to abundant preclinical evidences, limited numbers of clinical trials have been conducted to evaluate the antioxidant activities of algae in humans (8). A trial conducted in 2008 showed that type 2 diabetes patients had their serum interleukin 6 (IL-6) and MDA levels significantly reduced after consuming *Spirulina* for 12 weeks (84).

1.3.1.2 Anticancer properties of algae

Cancer, including tumorigenesis and metastasis, is a direct consequence of abnormal or uncontrolled cell growth that leads to the invasion or spreading to other parts of the body (1). Identification of selective and effective agents against cancer cells is essential for cancer therapy (85). Algae consumption has been associated with anti-cancer effects. In addition, multiple active anti-tumor agents have been isolated from algae, especially marine algae (86,87).

The anticancer properties of algae were demonstrated in a human study on tobacco-induced oral leukoplakia, in which 57% of subjects with leukoplakia were in complete regression after *Spirulina fusiformis* supplementation. However, after stopping the supplementation, over half of the complete responders had the relapse (88). The anticancer activities of algae have also been demonstrated in animal and cell culture models. For example, the injection of *Spirulina* and *Dunaliella* extracts inhibited chemically-induced carcinogenesis in hamster buccal pouches (89), and selectively inhibitory activities of algae on tumor cell lines have also been observed (90). In addition, the inhibitory effects of algae, such as *Spirulina*, on the carcinogen metabolizing enzymes in the liver have been reported (91).

Diverse mechanisms contribute to the anticancer activities of algae, which could originate from algal compounds themselves or from their metabolites. Even though the endpoint of these anticancer algal compounds is to induce cell death of cancer cells, other biological

activities, such as antioxidant activity or immune stimulation, are also involved in the process (1). Algal compounds, such as polysaccharides, can stimulate cytotoxic T lymphocyte or natural killer cells. DNA damage caused by free radicals can activate p53 inside the cell nucleus, and then p53 will trigger a serial of activations and result in apoptosis. The secondary metabolites including phenolics can scavenge free radicals, thereby preventing DNA damage (46) .

1.3.1.3 Algae and cardiovascular disease

Cardiovascular diseases (CVD), which includes atherosclerosis, vascular dysfunction, platelet aggregation, and other inflammation-associated conditions, are the top leading cause of mortality in the United States. High blood pressure, hyperlipidemia and hyperglycemia are highly associated with CVD. Healthy lifestyle and dietary intervention, such as the consumption of phytochemicals, decrease the risk of CVD (92). Among many dietary approaches that may reverse the phenotypes of CVD, the consumption of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), especially omega-3 fatty acids, is considered as an effective approach to reduce cholesterol level and to correct dyslipidemic condition.

Algae are rich in PUFAs and other bioactive compounds. Numerous studies have suggested that the consumption of algae can lower both cholesterol level and blood pressure (93-95). Feeding hyperchloesterolemic rats with four different algae, including *Ulva sp*., *Hypnea charodies*, *Colpomenia sinuosa and Sargassum hemiphylum*) led to

higher levels of high-density lipoproteins (HDL) (96). Feeding *Scenedesmus* diet also lowered the cholesterol level (97). The similar effect was observed in human trials. The consumption of *Spirulina* supplement by the subjects with mild hyperlipidemia or hypertension for 8 weeks dramatically decreased total serum cholesterol level (98). Additional feeding *Spirulina* studies, even though differing in study design, sample size and patient conditions, also showed similar results of decreasing LDL level (99,100). The underlying mechanisms of cholesterol-lowering activities are not fully elucidated, but are likely contributed by multiple active components of algae. For example, phycocyanin, the water soluble protein in algae, can reduce serum total cholesterol level and increase serum HDL level (7). It has been shown that the extract of *Spirulina platensis* can bind bile acids and therefore reduce cholesterol by decreasing the enterohepatic recycling of bile acids. Oxidative stress is another important biomarker relating to the CVDs, cancers, and hepatic injuries. Increased lipoprotein oxidation is one of the major contributors to atherosclerosis. It has been widely studied that antioxidants can significantly decreased atherogenesis. Algae present several bioactive compounds that have antioxidant properties such as carotenoids, phenolic compounds, and some vitamins, which can help protect against CVD (101,102).

Limited information is available on the effects of algae on blood pressure. Considering that minerals are a key factor in hypertension, the roles of minerals in the associations between algae consumption and blood pressure are under investigations (103,104).

1.3.1.4 Anti-obesity effects of algae

Obesity, as a widespread metabolic disorder in the adults and children in the United States, is associated with a variety of diseases, including type 2 diabetes, metabolic syndrome, coronary heart disease and even cancer (105,106). Diet is a major contributing factor for obesity. Therefore, anti-obesity components have been sought from food and dietary supplements.

Supplementation of algae has been shown to increase lean mass, prevent fat deposition, especially the visceral fat, and normalize the impaired glucose level (107). Besides the algal components that can treat dyslipidemia and CVD (detailed in 1.3.1.3), other active components with anti-obesity activities include insoluble fibers and carotenoids. The protective effects of insoluble fibers are largely based on their activities to reduce the absorption of dietary lipids, while among carotenoids, fucoxanthin, a xanthophyll from brown algae, has strong anti-obesity activity. It has been shown that fucoxanthin can induce the expression of uncoupling protein 1 (UCP1) in white adipose tissue, a protein that plays an important role in thermogenesis, energy expenditure, and protection against oxidative stress, and is mainly expressed in brown adipose tissue (108). All these results suggest that algae could be a candidate source for functional food and dietary supplement for preventing and treating obesity.

1.3.1.5 Perspective on the applications of algae in food

Concerns on the health and safety issues associated with the consumption of processed food have led to the regulatory changes on food safety in the United States, Europe, and

other countries, such as the prohibition of using synthetic dyes in food (7). Due to their activities in lowering cholesterol, improving cardiovascular system, and protection against oxidative stress and obesity, algae are becoming a potential choice for natural additives in food, such as coloring agents, or stand-alone dietary supplements, which are commonly consumed as capsules or tablets (109).

Incorporation of algal biomass into traditional food products has some major challenges, such as dark green color, the fishy taste, and the odor. Even though the dark color could be masked, the taste and odor are difficult to remove (6). In addition, the consumer acceptability is also a challenge in some regions and countries. For example, algae have been used as food for long time in Asia, especially China and Japan. In contrast, the consumption of algae was historically less common in most Western countries (6). Therefore, the acceptance of algae as commercial food materials is expected to be easier in Asian countries (110).

The utilization of algae and algal components has been expanded in multiple areas, including new algae species and new commercial products. Marine diatom *Odontella aurita* is a new algae species that was made into a novel food through microalgal biotechnology (111). More importantly, algae have been incorporated into commercial products such as bread, pasta, noodles, yogurts, beverages and other products (112). For example, *Spirulina* and *Chlorella* were used in pharmaceutical applications (7). Furthermore, besides whole biomass, the bioactive compounds of microalgae, such as fatty acids and pigments, were also used as the additives in food industry (113).

Algae also serve as new components in animal feeding that aim to improve the growth performance, productivity and fertility. The benefits observed in humans, such as the effects on antioxidant and cardiovascular systems, are expected in animals. It has been reported that feeding a small portion of algae can positively regulate the physiological system of animals, particularly the immune system (114).

1.3.2 Toxic effects of algae

Structural diversity of algal compounds and nutrients are associated with functional diversity. Many of these functions lead to the health promoting effects of algae (detailed in 1.3.1), while other functions could result in toxic effects. In the process of developing and approving algae and algal products as functional food or dietary supplement, the toxicology tests and experiments have been conducted to examine their safety in humans, animals, and in vitro models. The results from these studies have revealed the toxic effects of algae.

1.3.2.1 Toxicology studies of algae in animals

The safety of *Spirulina* has been widely tested in animal experiments. Its potential sub-acute and chronical toxicities were determined by examining reproduction, lactation, mutagenicity, and teratogenicity over three generations of mice after feeding three doses (10, 20, 30%) of *Spirulina* (115,116). No adverse effects were observed in those studies.

In addition, feeding *S. obliquus* as supplement on rats for two weeks did not cause any significant toxicological effects (117-119). Similarly, feeding both male and female rats with *S. maxima* for 13 weeks at three different concentrations did not lead to clear differences in behavior, food and water intake between tested group and control group (118,119).

1.3.2.2 Toxicology studies of algae with humans

Algae were considered as an important source of food and nutrients for humans in 1950s and 1960s. Various human feeding studies were performed during that period, such as malnourished infants or mass feeding trials. The results from these studies were often contradictory. Some studies reported no negative effects after the subjects solely consumed algae for prolonged periods, while others reported discomfort, vomiting, nausea, and poor digestibility even with small amounts of algae. For example, in a study published in 1961, five healthy male subjects, 18 to 23 year-old, consumed an algal mixture in doses ranging from 0 to 500 grams per day. The algal mixture was prepared by cooking and drying a mixture of *Chlorella sp*. and *Scenedesmus sp*.. All subjects reported no side effects up to 100 grams, but the taste was not desirable. Abdominal discomfort appeared at the 200-gram level, accompanied with nausea, vomiting and hard stool (120). In a separate feeding study, the subjects consumed the diets containing 50, 100, and 150 g freeze-dried algae (a mixture of *Chlorella sp*. and *Scenedesmus sp*.) for three weeks. Blood, urine and feces samples were collected at the end of the trial (121). The groups consuming 50 and 100 g of algae showed negligible changes of metabolic parameters, but

the consumption of 150 g of algae negatively affected the health status of the subjects. The study concluded that the maximum daily consumption of algae was 100 g, which was confirmed by a separate study later (122).

In the study of using microalgae as a food supplements for overcoming malnutrition, positive effects of algal consumption (10 g) on hospitalized children were observed as they gained significantly higher body weight than other children with normal diet (123).

Overall, some dose responses of algae feeding have been observed in these small-scale human trials. These observations and conclusions need to be confirmed by the studies with a large sample size. In addition, the mechanisms behind these observations are largely unknown.

1.3.3 Challenges of analyzing algae-induced biological and metabolic effects

At present, more and more studies are focused on the nutritional benefits of algae consumption, especially their therapeutic applications, including anti-inflammatory, antioxidant, and cholesterol-lowering activities. While algae can offer potential benefits, there are a number of concerns need to be considered before we can change the food supply in beneficial ways (124). Algae contain lots of nutrients and nutritionally beneficial components, many of which are known, but many of which are not yet. The study of biological beneficial effects are largely based on its components, such as PUFAs are responsible for cholesterol-lowering effects, carotenoids for antioxidant and anti-obesity effects, and polysaccharides for anti-cancer effects. But further metabolic effects

of algae cannot be simply analyzed through algal components. Therefore, the importance of metabolomics study merges recently. Metabolomics is the systematic study of metabolome, the unique biochemical fingerprint of all cellular processes. The successful development of metabolite profiling induced by algae will give a better idea explaining the beneficial of algae consumption.

1.4 PRINCIPLES AND PRACTICE OF NUTRITIONAL METABOLOMICS

In order to examine the fundamental components of biological systems, which are genomes (complete genetic), transcriptomes (gene expression), proteomes (protein expression), and metabolome (metabolites), the corresponding analytical tools are genomics (125), transcriptomics (126), proteomics (127) and metabolomics (128). Compare to the changes in genes and proteins, which mainly indicate the potential of physiological changes, changes in metabolites can truly reflect the real end-points of physiological process and metabolic consequences (129). Nowadays, metabolomics has been widely used in various scientific fields, including human nutrition, drug metabolism, clinical research and environmental toxicology.

1.4.1 Introduction of metabolomics platform

Metabolomics is a systematic study of chemical processes involving metabolites, which plays an important role in systems biology, replenishing genomics, transciptomics and

proteomics (129,130). Analysis of metabolic phenotypes in metabolomics study not only shows the status of metabolic system under different intervention, but also provides a starting point to further explore the changes in proteins and genes (131). Therefore, metabolomics has found broad application. Most widely used analytical instruments for metabolomics are nuclear magnetic resonance spectroscopy (NMR), mass spectrometry (GC-MS and LC-MS), other methodologies including electrochemistry and infrared spectroscopy have also been adopted, but not that common (132-135).

Compare to MS, NMR is highly quantitative and reproducible, and capable of providing more structural information, but less sensitive for detecting low-abundance metabolites. Moreover, NMR sensitivity does not depend on metabolite pK_a or hydrophobicity, making NMR an excellent choice for broad-based analyses (136). In the contrast, MS has reached much higher sensitivity than NMR in detecting small-molecule metabolites and thus can provide more comprehensive information on metabolite profile. However, MS also has its drawbacks, such as the need for sample preparation of majority MS-based metabolomics analysis, irrecoverable sample loss during MS analysis, and the lack of automatic metabolite identification (129).

Although many metabolomics studies were conducted with NMR, an increasing number of studies based on MS technology have been published recently. According to the chemical properties of samples, several separation methods can be selected for metabolomics analysis, including capillary electrophoresis, gas chromatography, or liquid chromatography (137). Capillary electrophoresis is highly efficient in separating polar and charged compounds in electric field, but the limitation of this method is the lower capacity

in sample loading and poor sensitivity on non-polar compounds (138). GC and LC platform are more commonly used when analyze biological samples. GC is an excellent platform because of its advantage in resolution and reproducibility of chromatographic separations. However, due to its column, it requires multiple steps of sample preparation processes, which can significantly affect integrity of the sample metabolome (139). Therefore, LC is more commonly used in MS-based metabolomics owing to the better compatibility with water-based biofluids and tissue/cell extracts, and no further chemical derivatization processes generally required by GC. High performance liquid chromatography (HPLC) used to be the predominant LC instruments. However, with the development on ultra-performance liquid chromatography (UPLC) and ultra-high pressure liquid chromatography (UHPLC), they are capable of smaller particles, faster flow rate, and higher pressure than HPLC, which greatly improved chromatographic resolution and reduced the running time. After eluting from GC or LC, analytes need to become ionized before they can be detected by mass detector, such as time-of-flight (TOF) or Fourier transform (FT) in MS-based chemical analysis. Overall, high-resolution and reproducible LC-MS methods lay a foundation for subsequent data processing and multivariate data analysis (129,137).

1.4.2 Application of metabolomics on nutritional study

Metabolomics is capable of identifying novel metabolites of xenobiotics, elucidating biotransformation pathways, and investigating endogenous metabolism and disease mechanisms. Therefore, it has been widely used in pharmacology and toxicology, but is

relatively new in nutritional study (140). Thus, the increasing application of metabolomics to nutrition research from assessing novel biomarkers of dietary intake to metabolomics in intervention studies will better enhance our understanding of the role of food in health and disease (141).

1.4.2.1 Sample preparation and LC-MS analysis

In order to maximize the information available for LC-MS analysis, sample preparation needs to be designed and performed based on chemical and biochemical properties. Without appropriate processing, biological samples such as urine, blood, and tissue are not suitable for LC-MS analysis due to the biomatrices of the samples. Removing proteins and particles are required for all samples during basic sample preparation procedures, while other specific procedures are adopted according to the physical and chemical properties of samples as well as the aims of metabolomics analysis (129). Sample preparation not only makes samples more compatible for LC-MS analysis, but also enhances the sensitivity of metabolite detection (142).

The major components of LC-MS analysis are the mobile phase and column of the LC system, and the ion source and mass detector of the MS system, which can significantly affect data quality. The mobile phase is critical to metabolite separation in the LC column and also facilitates metabolite ionization in the MS system (143). In addition to choosing appropriate solvents (such as acetonitrile, methanol, water) and solvent gradients based on the chemical properties of the samples, the addition of eluent additives to suppress

unwanted signals or selectively enhance signals of interest can greatly increase the ability to detect the sensitivity to quantify particular compounds in a mixture.

Ionization is a prerequirment for mass detection in MS system. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), which are commonly used in LC-MS analysis, together with the chemical components of mobile phase, can have major impacts on ionization efficiency (137). Generally, for mass detection, the triple quadrupole MS can perform highly selective and sensitive quantitation of targeted metabolites, while detectors with full scan and accurate mass measurement capacities such as TOF or Fourier transform MS are more suitable for untargeted metabolites because of their high resolution of acquire accurate mass for elemental composition analysis and their high capacity to detect multiple ions simultaneously for comprehensive metabolite profiling (144).

1.4.2.2 Data processing and analysis

Chromatographic and spectroscopic data generated from LC-MS analysis need to be properly processed before being used in multivariate data analysis (MDA). General processes include data condensation and reduction by centroiding and deisotoping mass spectra; removal of noise or background signals; and peak identification by setting threshold windows for mass-charge ratio (m/z) and retention time (RT) (145). Normalization of MS data against parameter of the whole data set (such as total ion count, median ion count) or intensities of internal standards (such as creatinine in urine)

is also commonly conducted to reduce the influence of systematic and sample biases (such as dilution or condensation) (129). Each pair of RT-m/z is unique and therefore can act as the identity of one compound. Overall, the processed datasets can be either directly used for MDA or further statistically transformed and scaled according to the properties of data and the purpose of MDA.

Unsupervised MDA, such as principal components analysis (PCA) and supervised MDA, such as projection to latent structures discriminant analysis (PLS-DA) or orthogonal PLS (OPLS) are the two major categories of MDA methods during metabolomics data analysis. Compared to the traditional statistical methods, such as t-test and ANOVA, MDA can better handle and interpret the large datasets generated by LC-MS analysis, in which, a model is represented by the one or multiple principle components (PC) to explain a large portion of examined datasets (146). The established MDA model and its PCs can be presented in the scores plot, where sample-PC and sample-sample relationships can be visualized, reflect their differences in chemical composition. When a clear separation between samples is observed in the score plot, the contribution of individual ions to PCs and to the group separation can be further examined in the loadings plot (137).

1.4.2.3 Identification of novel metabolites and metabolism pathways

The combination of LC-MS and MDA has been applied and showed very efficient in identifying metabolites of foods and drugs, and elucidating *in vivo* metabolic pathways

(147-149). Biological samples (urine, serum or tissues) collected after parallel control diet intake can be used in identifying *in vivo* dietary metabolites, as to compare with LC-MS chromatograms. The chemical identities of biomarkers and interested metabolites can be determined by accurate mass measurement, elemental composition analysis, MS/MS fragmentation and subsequent database search. But sometimes, it is difficult to identify in vivo metabolites from thousands of chemical species only through visual examination of LC-MS chromatograms and fragmentation analysis, thus, a stable isotope-based metabolomics approach is proposed to facilitate the identification of xenobiotic metabolites and endogenous metabolites (**Figure 1.6**). With appropriate processing procedures, the separation of vehicle-treated and xenobiotic-treated sample groups can be achieved in the scores plot of a multivariate model, and xenobiotic metabolites ions can be conveniently identified by analyzing ions contributing to the separation of xenobiotic treatment from vehicle treatment (150).

Overall, the wide adoption of metabolomics in biomedical research in recent years has proved its advantages and importance over traditional metabolites analysis methods. LC-MS –based metabolomics techniques combine sensitivity and selectivity, possess a great promise of becoming the most commonly tools to identify novel metabolites and elucidate metabolic changes. Although there are still limitations and unsolved problems, with the development of new LC-MS techniques and data analysis methods, LC-MS-based metabolomics will have more applications in both exploratory and hypothesis-driven investigations.

Table 1.1 Macronutrient contents in algae and common human food (% of dry matter)

	Carbohydrate	Lipid	Protein	Nucleic acid
Spirulina maxima	13-16	6-7	60-71	3-4.5
Spirulina platensis	8-14	4-9	46-63	2-5
Chlorella vulgaris	12-17	14-22	51-58	4-5
Scenedesmus obliquus	10-17	12-14	50-56	3-6
Dunaliella salina	32	6	57	
Synechococcus sp.	15	11	63	
Cow milk	38	28	26	
Meat muscle	1	34	43	
Soybean	30	20	37	

Table 1.2 A comparison of amino acid compositions of algae species with egg and RDIs recommended by FAO and WHO

	WHO/FAO[a]	Egg[a]	*Spirulina maxima*[a]	*Spirulina platensis*[a]	*Chlorella vulgaris*[a]	*Dunaliella bardawil*[a]	*Scenedesmus obliquus*[a]
Ala	—	—	6.8	9.5	9.4	7.3	9
Val*	5	7.2	6.5	7.1	7	5.8	6
Ile*	4	6.6	6	6.7	3.2	4.2	3.6
Leu*	7	8.8	8	9.8	9.5	11	7.3
Phe*	6.3	5.8	4.9	5.3	5.5	5.8	4.8
Tyr	6	4.2	3.9	5.3	2.8	3.7	0.3
Try*	1	1.7	1.4	0.3	—	0.7	0.3
Met*	2.9	3.2	1.4	2.5	1.3	2.3	1.5
Cys	3.5	2.3	0.4	0.9	—	1.2	0.6
Pro	—	4.2	3.9	4.2	5	3.3	3.9
Asp	—	11	8.6	11.8	9.3	10.4	8.4
Glu	—	12.6	12.6	10.3	13.7	12.7	10.7
Gly	—	4.2	4.8	5.7	6.3	5.5	7.1
Ser	—	6.9	4.2	5.1	5.8	4.6	3.8
Thr*	4	5	4.6	6.2	5.3	5.4	5.1
Lys*	5.5	5.3	4.6	4.8	6.4	7	5.6
Arg	—	6.2	7.4	7.3	6.9	7.3	7.1
His*	—	2.4	1.8	2.2	2	1.8	2.1

*: essential amino acids
WHO/FAO: Essential amino acid requirements for adults
a: g per 100 g protein

Table 1.3 Fatty acid composition in selected algae species (% in total fatty acids)

	Spirulina maxima	*Spirulina platesis*	*Chlorella pyrenoidosa*	*Chlorella vulgaris*
C12:0	0.52	0.84	1.07	0.38
C14:0	N/A	N/A	0.58	0.69
C16:0	35.82	42.30	14.60	15.41
C16:1	0.85	1.00	3.07	1.17
C16:2	4.61	2.43	5.10	N/A
C16:3	N/A	N/A	3.16	N/A
C18:0	1.49	0.95	1.93	6.24
C18:1	5.03	1.97	18.24	33.14
C18:2	16.34	16.18	11.24	9.73
C18:3 (ω-3)	N/A	N/A	15.87	1.93
C18:3 (ω-6)	18.16	20.06	N/A	N/A
C20:0	N/A	N/A	N/A	0.19
C20:1	N/A	N/A	N/A	N/A
C20:2	0.59	0.96	0.71	N/A
C20:5	N/A	N/A	0.40	3.23
Saturated	46.31	51.64	33.00	22.22
Monounsaturated	11.24	5.88	23.98	35.44
polyunsaturated	40.36	39.51	36.48	38.94

Table 1.4 A comparison of vitamin levels among algae, beef liver, spinach, (mg/kg dry matter) and RDI (mg/day)

	RDI (mg/day)	*Spirulina platensis*	*Spirulina maxima*	*Scenedesmus obliquus*	*Chlorella pyrenoidosa*	Beef liver	Spinach
Vitamin A/caroteinoids	1.7	840	225	230	480	360	130
Vitamin B1	1.5	44	14	8	10	3	0.9
Vitamin B2	2.0	37	28.5	36.6	36	29	1.8
Vitamin B6	2.5	3	1.3	2.5	23	7	1.8
Vitamin B12	0.005	7	0.3	0.4	0.02	0.65	—
Vitamin C	50	80	103	20	—	310	470
Vitamin E	30	120	—	—	—	10	5.5
Nicotinate	18	—	—	120	240	136	0.07
Biotin	—	0.3	—	0.2	0.15	1	0.7
Folic acid	0.6	0.4	—	0.7	—	2.9	2.8
Pantothenic acid	8	13	—	16.5	20	73	—

Table 1.5 List of common algal toxins and their mechanisms.

Toxin	Algal species	Targeted tissue	Mechanism
Cyanotoxins	*Microcystis sp.* *Nodularia sp.* *Aphanizomenon sp.*	Liver	Inhibition of protein phosphatases type 1 and 2A
	Anabaena sp. *Oscillatoria sp.*	Nervous system	Irreversible inhibitor of acetylcholinesterase
Domoic acid	*Pseudonitzschia sp.*	Nervous system	Activation of glutamate receptors
Saxitoxin	*Alexandrium sp.*	Nervous system	Sodium channel blocker
Brevetoxin	*Gymnodinium sp.*	Nervous system	Activation of voltage-sensitive sodium channel

Figure 1.1 Classification of algae

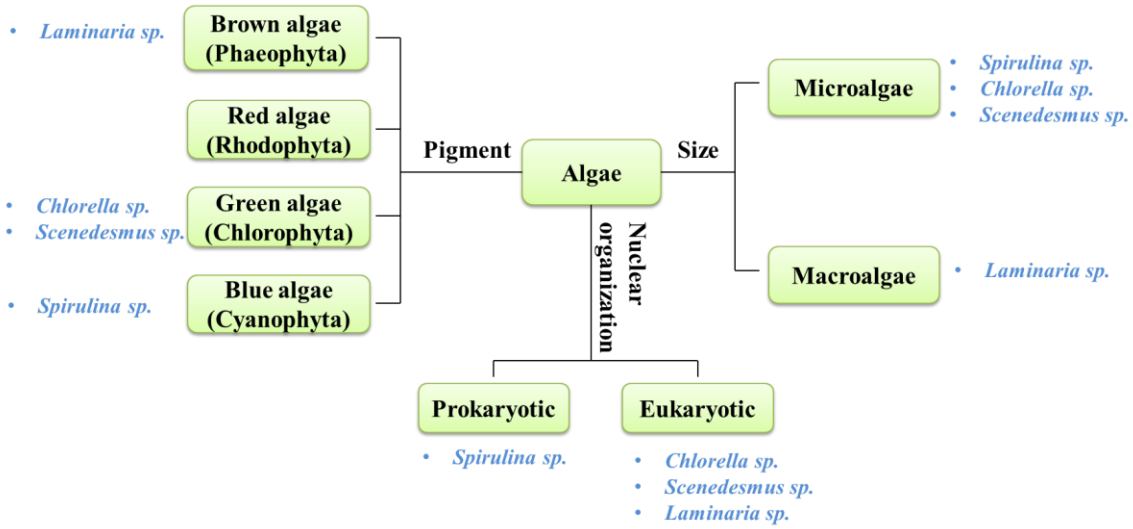


Figure 1.2 Summary of production and utilization of algae

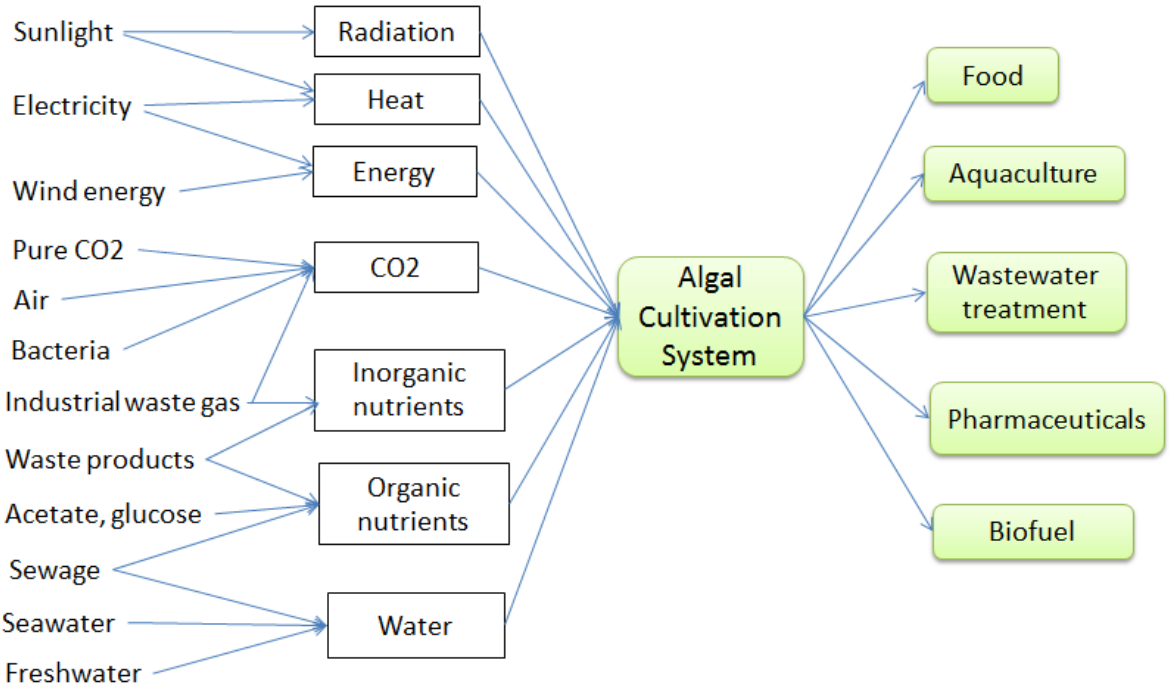
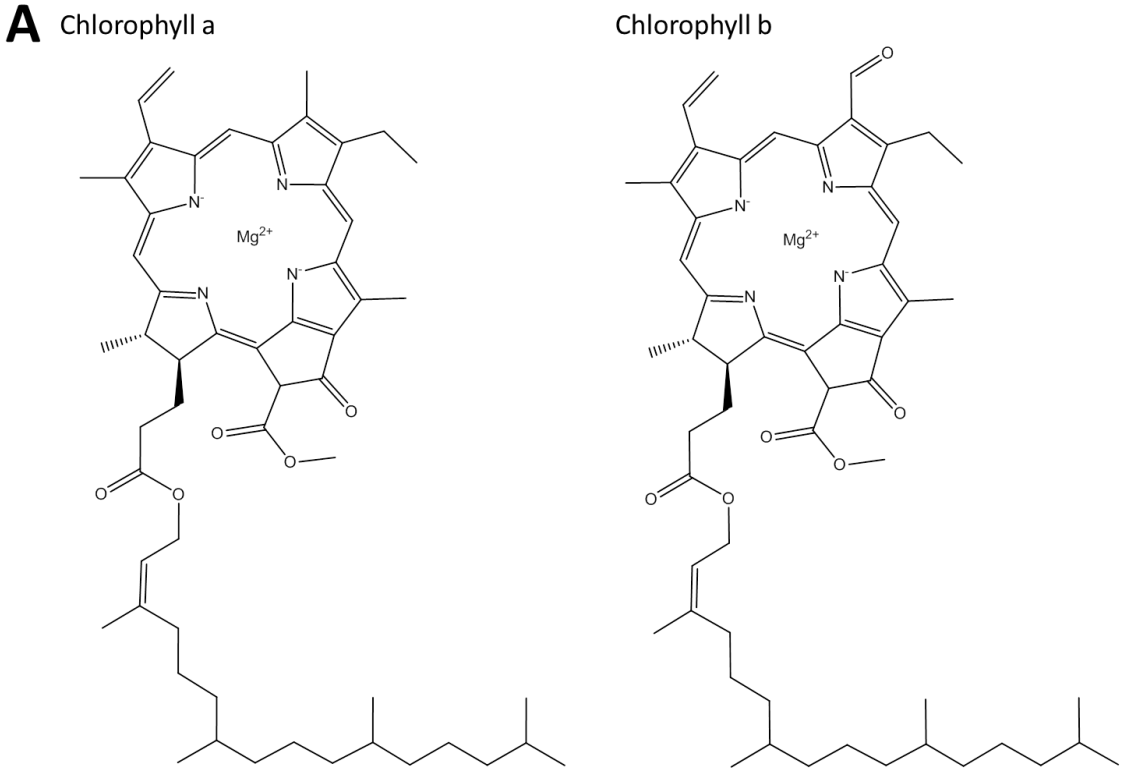
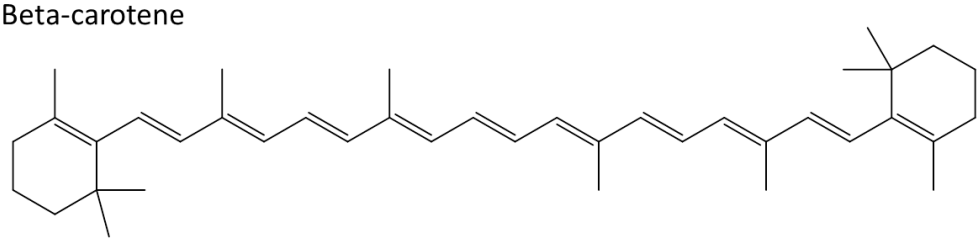


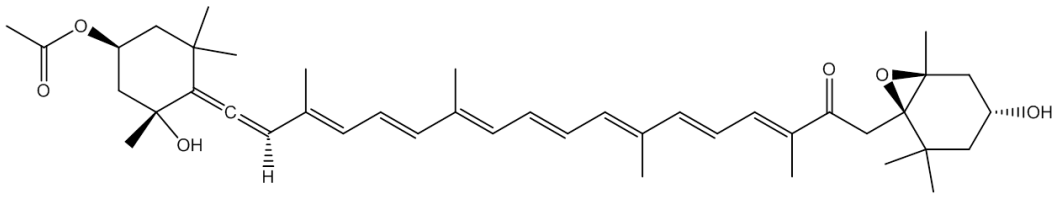
Figure 1.3 Chemical structures of important algal compounds. ***A.*** Chlorophylls. ***B.*** Carotenoids. ***C.*** Phycobiliproteins



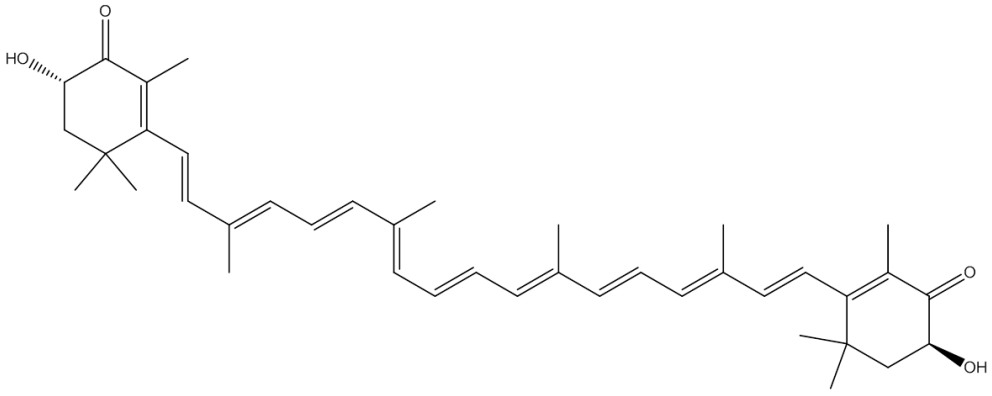
B Beta-carotene



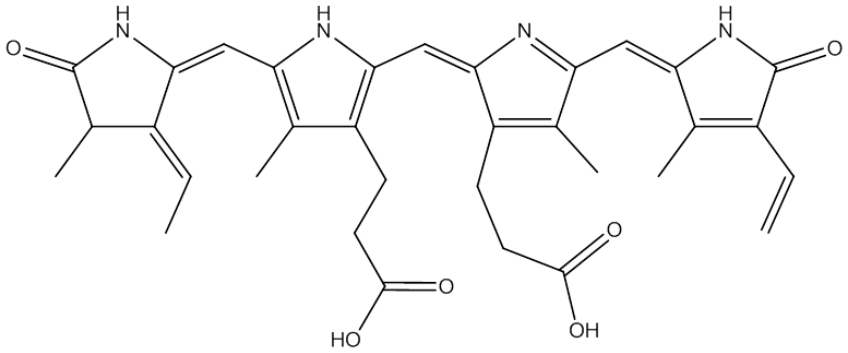
Fucoxanthin



Astaxanthin



C Phycoerythrobilin



Phycocyanobilin

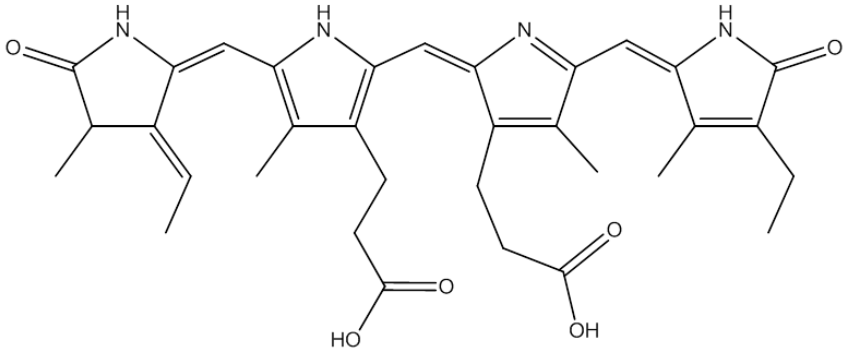


Figure 1.4 Synopsis of algae inhibition in free radical formation, oxidative stress and pathogenesis of chronic disease

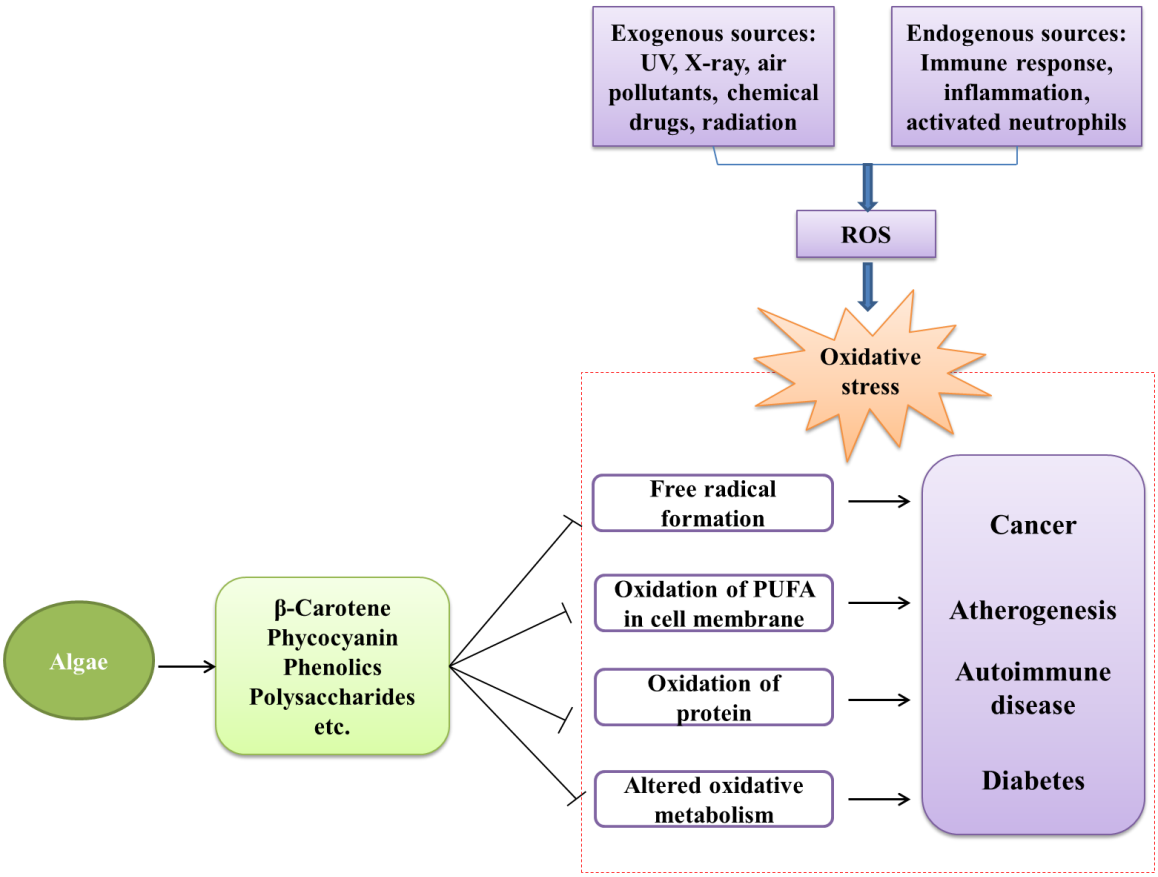


Figure 1.5 Exploratory and hypothesis-driven metabolomics

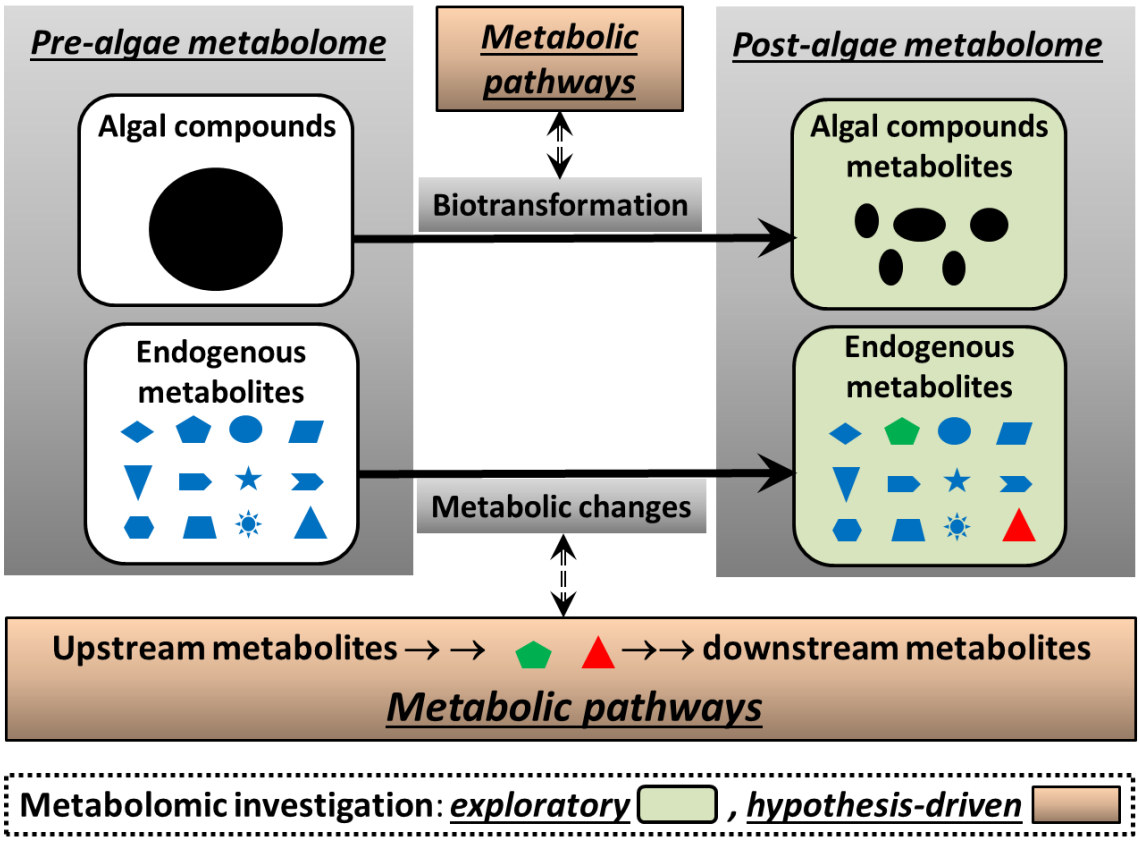
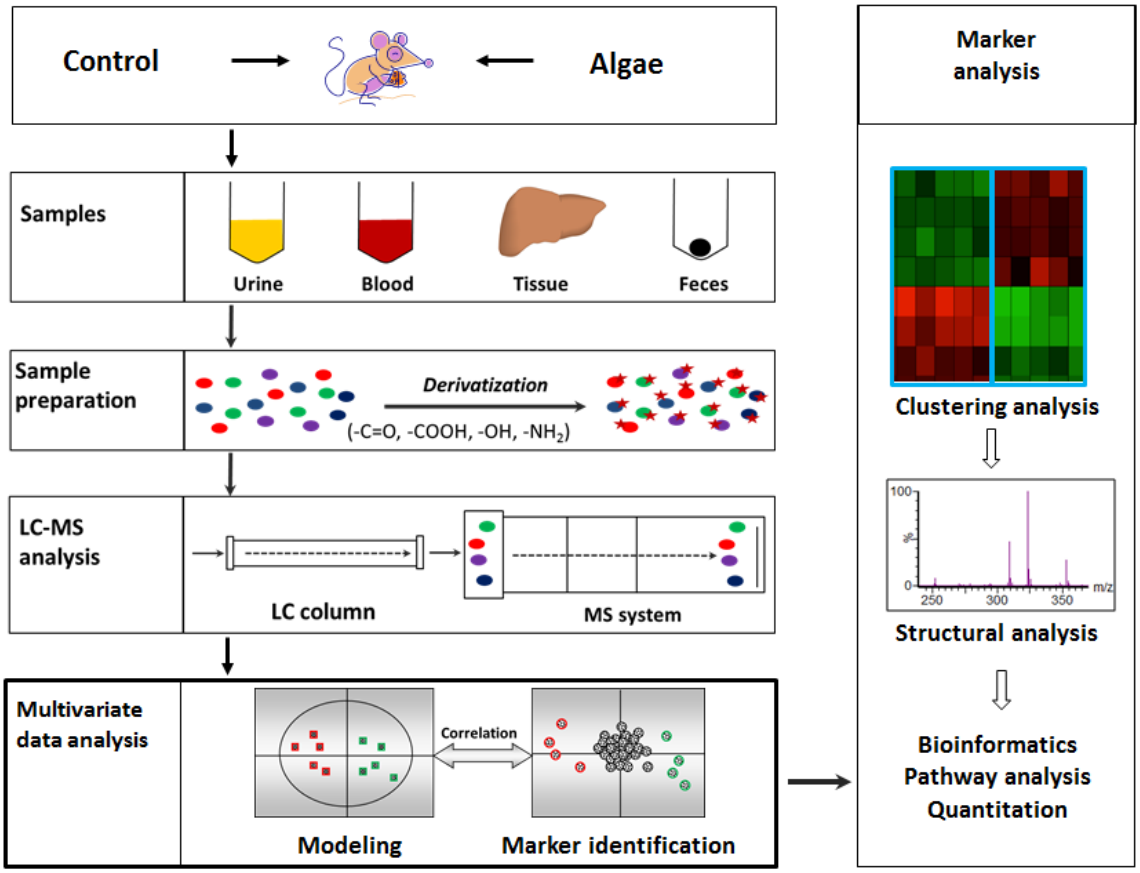


Figure 1.6 The work flow of LC-MS-based metabolomics data analysis



CHAPTER 2 METABOLOMICS ANALYSIS REVEALED DOSE-DEPENDENT CORRELATIONS BETWEEN GROWTH PERFORMANCE AND ALGAE FEEDING-INDUCED METABOLIC CHANGES IN MOUSE

[2]**KEYWORDS:** microalgae, growth performance, exposure markers, redox balance, lipidomics, microbial metabolism.

ABBREVATION: ACN, acetonitrile; ADFI, average daily food intake; ADG, average daily growth; ALT, alanine transaminase; AST, aspartate transaminase; BUN, blood urea nitrogen; CoA, coenzyme-A; DC, dansyl chloride; DHA, docosahexaenoic acid; DPDS, 2,2'-dipyridyl disulfide; EPA, eicosapentaenoic acid; ESI, electrospray ionization; FAA, free amino acid; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; GSH, glutathione; GSSG, oxidized glutathione; HCA, hierarchical clustering analysis; HQ, 2-hydrazinoquinoline; LC-MS, liquid chromatography-mass spectrometer; LCA, lithocholic acid; LOP, lipid oxidation product; MDA, malondialdehyde; NAD, nicotinamide adenine dinucleotide; PC, principal component; PCA, principal component analysis; PE, phosphoethanolamine; PLS-DA, partial least squares-discriminant analysis; PUFA; polyunsaturated fatty acid; QTOF, quadrupole time-of-flight mass spectrometer; SCFA, short chain fatty acid; SIC, single ion counts; TAG, triacylglycerol; TCA, taurochoilc acid; TCDCA, taurochendeoxycholic acid; TIC, total ion counts; TMCA, tauromuricholic acid; TPP, triphenylphosphine; UPLC, ultra-performance liquid chromatography.

2.1 INTRODUCTION

Green algae have greater efficiency than many crop plants in solar energy harvesting, carbon dioxide fixation, and nutrient production, leading to extensive exploration of green algae as a potential staple source of human food in 1940s and 50s (151). Because of technical difficulty and economic considerations on large-scale green algae farming, this effort of human food production has been largely suspended (14). However, the use of green algae as dietary supplement in humans remains very common due to the fact that green algae are an enriched source of nutrients, including protein, essential amino acids, vitamins, minerals, and polyunsaturated fatty acids (PUFA) (152,153), and bioactive chemicals, such as carotenoids, astaxanthin, phycobiliproteins and insoluble fiber (154,155). These nutrients and bioactive chemicals not only support energy metabolism and biomass production, but also are responsible for antioxidant, anti-inflammatory, and hypolipidemic activities of green algae (8).

Scenedesmus is one of the most common freshwater green algae genera (156). Known for their capacity in photosynthesis, lipid accumulation and nutrient removal, the species in this genera have been widely selected for biodiesel production and waste water treatment (157,158). The biomass produced from these applications has been explored as an animal feed ingredient (159-161). Considering diverse nutrients and bioactive chemicals in *Scenedesmus* and other green algae, animals are expected to undergo many changes or even challenges in their metabolic systems after feeding and exposure. However, current knowledge on the metabolic effects of algae feeding is limited to growth performance and

several specific biochemical parameters of blood lipids including cholesterol and triglyceride levels, oxidative stress, and immune responses, mainly from feeding *Spirulina* and *Chlorella* algae (8,162-166). A comprehensive investigation on the metabolic effects of feeding *Scenedesmus* algae has not been reported.

In this study, besides growth performance and blood chemistry, liquid chromatography-mass spectrometry (LC-MS)-based metabolomic analysis of serum, hepatic extract, fecal extract, and urine samples was performed to define the metabolic effects induced by feeding dried *Scenedesmus* algae to young mice. The metabolite markers associated with dose-dependent effects of algae feeding were identified and characterized.

2.2 MATERIALS AND METHODS

2.2.1 Culture of Scenedesmus algae

Scenedesmus spp. (UMN 285), a species isolated locally in Minnesota, was chosen as the source of green algae in the feeding experiment for its nutrient content. In a separate screening study, the level of eicosapentaenoic acid (EPA) in UMN285 was found to be relatively higher than its levels in other examined local green algae species (167). To culture UMN 285, the alga seeds were first inoculated into autoclaved BG-11 medium containing 2 g/L glucose, and then kept at 25 ± 2^{o}C on a shaker at 100 rpm under a continuous cool-white fluorescent light illumination at 100 μmol $m^{-2}s^{-1}$. After 1-2 week

batch cultivation, algae were separated from the culture broth by centrifugation at 500 × g for 10 min and were washed with deionized water. This centrifugation-washing process was repeated twice. Harvested algae were dried for further use (168,169).

2.2.2 Chemicals

LC-MS-grade water and acetonitrile (ACN) were purchased from Fisher Scientific (Houston, TX). 2-Hydrazinoquinoline (HQ) and triphenylphosphine (TPP) were purchased from Alfa Aesar (Ward Hill, MA). 2-2'-Dipyridyl disulfide (DPDS) were purchased from MP Biomedicals (Santa Ana, CA). Dansyl chloride (DC) and n-butanol were purchased from Sigma-Aldrich (St. Louis, MO). Standards of identified metabolites were purchased from Sigma-Aldrich, Fisher Scientific, Alfa Aesar, Ark Pharm (Libertyville, IL), Frontier Scientific (Logan, UT), and Steraloids (Newport, RI), respectively.

2.2.3 Standard synthesis

Basically, 4-methyl pentanoic acid or 4-methyl hexanoic acid was dissolved in thionyl chloride to synthesis fatty acid chlorides. The mixture was incubated at 80^{o}C for 30 min and then was added into glycine, which suspended in dry pyridine. The reaction mixture was incubated at 4 ^{o}C for 24hrs with continuously stirring, and then acidified with 80 µl of 12M HCl. After 10 minutes centrifugation at 18000 × *g*, the supernatant was collected and diluted 1000 times for LC-MS analysis.

2.2.4 Animal treatment and sample collection

Male C57BL/6 mice (n=24), 8 weeks old, were purchased from Charles River Lab (Wilmington, MA). All mice were housed in the University of Minnesota animal facility at a constant temperature of 21^{o}C under a 12 h light/dark cycle and had access to water and feed *ad libitum*. Handling and treatment procedures were in accordance with animal study protocols approved by the UMN Animal Care and Use Committee. Mice were first acclimated to the AIN93G diet for 7 days, and then randomly divided into three different groups. First group of mice (n=8) continued to be fed the control AIN93G diet. Second group (n=8) was fed a 5% algae diet, composed of 95% AIN93G and 5% dried algae powder. Third group (n=8) was fed a 20% algae diet, composed of 80% AIN93G and 20% algae powder. Diet was made into pellet with 7-8% water content.

On day 26 of feeding experiment, urine and fecal samples were collected by housing animals individually in the metabolic cages for 24 h. On day 28, blood samples were collected by submandibular bleeding, and tissue samples were collected after carbon dioxide euthanization. All samples were stored at -80^{o}C until further analysis.

2.2.5 Growth performance measurement

Mice were weighed individually every day in the first seven days since the dietary treatment started and three times a week after the acclimation period to calculate average daily growth (ADG). On each weight day, amount of food disappearance was measured to calculate average daily food intake (ADFI). ADG and ADFI were used to calculated gain/feed ratio.

2.2.6 Serum biochemical analysis

Serum cholesterol, triacylglycerol (TAG), glucose, and blood urea nitrogen (BUN) concentrations were determined using respective colorimetric assay kits from Pointe Scientific (Canton, MI) in a Spectramax 96-well plate reader (Spectra Max 250, Molecular Devices, Sunnyvale, CA).

2.2.7 Sample preparation for LC-MS analysis

Serum, urine, liver, and fecal extracts were examined by LC-MS-based metabolomic analysis, which comprises sample preparation, chemical derivatization, LC-MS analysis, data deconvolution and processing, multivariate data analysis, and marker characterization.

Urine was diluted with 5 volume 50% aqueous acetonitrile and centrifuged at 18,000 × g for 10 min to remove the proteins and particles. Fecal samples were diluted with 50

volumes of 50% aqueous acetonitrile overnight. Extract by vortexing and sonication for 10 min and centrifuge at 18,000 × *g* for 10 min to remove the solid fraction and particles. Serum samples were conducted by mixing one volume of serum with 19 volumes of 66% aqueous ACN and then centrifuging at 18,000 × *g* for 10 min to obtain the supernatants. Liver extraction was extracted based on the principle of Bligh and Dyer method (170). Briefly, 100 mg of frozen liver sample was homogenized in a mixture of 0.5 ml methanol, 0.5 ml chloroform and 0.4 ml water. After 10 min centrifuge at 18,000 × g, fractions were separated. Aqueous fraction was stored at -80ºC, lipid fraction in chloroform was dried under nitrogen and reconstituted in 0.5 ml n-butanol.

2.2.8 Chemical derivatization

For detecting the metabolites containing amino functional group in their structures, the samples were derivatized with dansyl chloride (DC) prior to the LC-MS analysis. Briefly, 5 µl sample or standard was mixed with 5 µl of 100 µM p-chlorophenylalanine (internal standard), 50 µl of 10 mM sodium carbonate, and 100 µl of DC (3 mg/mL in acetone). The mixture was incubated at 60ºC for 15 min and centrifuged at 18,000 × *g* for 10 min, the supernatant was transferred into a HPLC vial for LC-MS analysis. For detecting carboxylic acids, aldehydes and ketones, the samples were derivatized with HQ prior to the LC-MS analysis. Briefly, 2 µl of sample was added into a 100 µl of freshly-prepared ACN solution containing 10 mM DPDS, 10 mM TPP and 10 mM HQ. The reaction mixture was

incubated at 60°C for 30 min, and then immediately chilled on ice and then mixed with 100 µl of ice-cold H_2O. After centrifugation at 18,000 × g for 10min, the supernatant was transferred into a HPLC vial for LC-MS analysis.

In order to detect the concentration of fatty acids in plasma sample, both FA standards and plasma samples underwent the same extraction, hydrolysis, and derivatization steps before introduced into LC-MS system. The derivatization procedure was modified based on the previous study Li's method (171). In brief, 5 µl samples were mixed with 100 µl MeOH, which containing 50 µM internal standard and 17.5 µl of 40% aq. KOH. Mixture was vortexed and heated at 60°C for 30 min, followed by buffering with 100 µl of aq. Phosphate buffer (75 mM, pH7) and acidifying with 30 µl of aq. 2.5M HCl. Vortex the mixture again for 1 min and take 2 µl supernatant out to continuous with HQ derivatization.

2.2.9 LC-MS analysis

The analysis was performed by a Water Acquity ultra-performance liquid chromatography (UPLC) system (Milford, MA). A 5 µl aliquot of diluted urine, fecal, serum or liver aqueous fraction samples was injected into an Acquity ultra-performance liquid chromatography (UPLC) system (Water, Milford, MA) and separated in a BEH C 18 column with a gradient of mobile phase ranging from water to 95% aqueous acetonitrile consisting of 0.1% formic acid in a 10-minute run. The LC eluate was directly introduced into a Waters QTOF mass spectrometer for the accurate mass measurement and ion

counting. Capillary voltage and cone voltage for electrospray ionization (ESI) was maintained at 3 kV and 30 V for positive-mode detection, or at -3 kV and -35V for negative-mode detection, respectively. Nitrogen was used as both cone gas (50 liters/h) and desolvation gas (600 liters/h) and argon as collision gas. For accurate mass measurement, the mass spectrometer was calibrated with sodium formate solution (range *m/z* 50-1000) and monitored by the intermittent injection of the lock mass leucine encephalin ($[M+H]^+$ = *m/z* 556.2771 or $[M+H]^-$ = *m/z* 554.2615). Mass chromatograms and mass spectral data were acquired and processed by MassLynx^{TM} software (Waters) in centroided format. Additional structural information was obtained by tandem MS (MS/MS) fragmentation with collision energies ranging from 15 to 45 eV.

2.2.10 Multivariate data analysis

Chromatographic and spectral data of samples were analyzed using MarkerLynx software (Waters). A multivariate data matrix containing information on sample identity, ion identity [retention time (RT) and *m/z*], and ion abundance was generated through centroiding, deisotoping, filtering, peak recognition, and integration. The intensity of each ion was calculated by normalizing the single ion counts (SIC) versus the total ion counts (TIC) in the whole chromatogram. The data matrix and sample list were further exported into SIMCA-P+ software (Umetrics, Kinnelon, NJ). Principal components analysis (PCA) and projection to latent structures-discriminant analysis (PLS-DA) were generated after

data were transformed by mean-centering and Pareto optimization to analyze the data from control and algae-treated C57BL/6 mice. Major latent variables in the data matrix were described in a scores scatter plot of multivariate model. The potential metabolites after algae feeding were identified by analyzing ions contributing to the principal components and to the separation of sample groups in the loadings scatter plot. The metabolite structures were identified by accurate mass measurement, elemental composition analysis, database search (Human Metabolome Database: http://www.hmdb.ca/, Lipid Maps: http://www.lipidmaps.org/), MS/MS fragmentation, and comparison with authentic standards if possible.

2.2.11 Marker characterization and quantification

The chemical identities of interested compounds were determined by accurate mass measurement, elemental composition analysis, database search using MassTRIX serch engine (http://masstrix3.helmholtz-muenchen.de/masstrix3/), MSMS fragmentation, and comparisons with authentic standard if available. Individual metabolite concentrations were determined by calculating the ratio between the peak area of metabolite and the peak area of internal standard and fitting with a standard curve using QuanLynx^{TM} software (Waters).

The concentrations of GSH and GSSG in liver were measured by LC-MS. Briefly, samples for total GSH and reduced GSH measurement were prepared by homogenizing liver in 10

volumes of 5% 5-sulfosalicylic acid. Precipitated protein was removed by centrifugation at 18,000 × *g* for 10 min, and supernatant was diluted by deionized water prior to LC-MS analysis.

2.2.12 Statistical analysis

The data were tested for normality first and then processed by logarithm, square root, or inverse transformations if needed. Statistical analysis was performed by one-way ANOVA and Tukey – Kramer comparison test using the PROC GLM procedure of SAS version 9.1 (SAS Institute, Cary, NC). Data are reported as least squares mean (LSMEANS) and the differences are considered significant if $P < 0.05$.

2.3 RESULTS

2.3.1 General responses to algae feeding

Young mice in their growing phase are generally considered to be sensitive to the nutritional conditions (172).The C57BL/6 mice used in this study were 8 weeks old prior to the feeding of three experimental diets, i.e. control AIN93G, 5% algae, and 20% algae. After 28 days of feeding, significant differences in body weight gain and feed intake were observed among three groups of mice. Compared to the control group, feeding 5% algae

increased the average daily gain (ADG) (*P<0.01*) whereas 20% algae decreased it (*P<0.05*) (**Fig 2.1A).** Average daily feed intake (ADFI) was increased by 5% algae, but not affected by 20% algae (**Figure 2.1B**). The feeding efficiency, indicated by the ratio between the growth and the feed intake (G:F ratio), was significantly decreased by 20% algae (**Figure 2.1C**). Furthermore, 20% algae feeding might result in polyuria, as the volume of 24-h urine produced by the mice under 20% algae was 2-3 folds higher than that from the control and 5% algae feeding. Overall, these observations suggested that feeding 5% algae promoted the growth while 20% algae was associated with negative responses.

2.3.2 Effect of algae feeding on blood chemistry

Serum glucose, urea, triacylglycerols (TAG), and cholesterol levels partially reflect the general status of macronutrient metabolism. The results from blood chemistry tests showed that neither 5% nor 20% algae feedings affected the serum concentrations of glucose, and blood urea nitrogen (BUN) (**Figure 2.2A-B**). However, 4 weeks of 20% algae feeding significantly decreased both serum triacylglycerides and cholesterol concentration as compared to the control group (**Figure 2.2C-D**). This decrease of serum cholesterol was not associated with liver injury since the activities of alanine transaminase (ALT) and aspartate transaminase (AST) in serum were not affected by algae feeding (**Figure 2.2E-F**).

2.3.3 Metabolomic investigation of algae-induced metabolic events

In order to investigate the metabolic events induced by algae feeding, the urine, fecal, serum and liver extract samples from three groups of mice were examined through LC-MS-based metabolomic analysis. Besides adopting optimized mobile phase, ionization condition and MS parameters, efficient chromatographic separation and sensitive spectroscopic detection of diverse metabolite in these samples were also facilitated by chemical derivatization in sample preparation, such as dansylation for amino-containing metabolites and 2-hydrazinoquinoline derivatization for carboxylic acids, aldehydes and ketones (173). After processing the data acquired from the LC-MS analysis by principal components analysis (PCA), multivariate models were constructed to determine the metabolic differences among the control and algae feeding groups. As shown by the scores plots, dose-dependent separation of four types of samples were observed in four PCA models, respectively, suggesting that algae feeding altered the mouse metabolome in a dose-dependent pattern (**Figure 2.3A-D**). More importantly, the separation of control and 20% algae occurred along the first component of the models, while 5% algae differed from control and 20% algae in the secondary component of the models. This pattern of sample distribution in the PCA models suggested that 5% and 20% algae feedings had different influences on mouse metabolome. Subsequently, the metabolites associated with 5% and 20% algae feedings were identified in the loadings plots of PCA models, and their chemical identities were defined by elemental composition analysis, database search, MSMS fragmentation, and confirmation by authentic standards. Overall, more than 120

metabolites in different biological samples (liver, feces, serum, and urine) were identified as algae-responsive metabolites (**Table 2.1**). Based on their origins and biochemical properties, these metabolite markers were further categorized as the indicators of algae exposure, amino acid metabolism, lipid metabolism, redox balance, and microbial metabolism.

2.3.3.1 Structural elucidation of metabolite markers

Multiple approaches were used to determine the structures of the metabolites affected by algae feeding, especially for the ones without authentic standards. For example, the fatty acid compositions of triacylglycerol (TAG) markers, such as TAG (18:2/20:4/20:5), were elucidated by analyzing the fatty acid fragments in MSMS fragmentograms of fatty acid-ammonium adducts (**Figure 2.4A**). The structures of phosphatidylcholines (PC), such as PC (16:0/20:5), were determined by detecting the phosphocholine fragment in positive-mode MSMS fragmentograms and the fatty acid fragments in negative-mode MSMS fragmentograms (**Figure 2.4B**). Moreover, the structures of two branched fatty acid metabolites in urine, glyco-4-methyl-pentanoic acid and glyco-4-methyl-hexanoic acid, were confirmed by synthesizing the glycine conjugates of 4-methyl-pentanoic acid and 4-methyl-hexanoic acid and comparing their MSMS fragmentograms with corresponding urinary metabolites (**Figure. 2.4C-D**).

2.3.3.2 Characterization of algae exposure markers

As exogenous compounds, vitamins and phyto-pigments can function as effective markers of algae exposure. The PLS-DA model on the urine samples from three feeding groups revealed the higher levels of water-soluble B vitamins after algae feeding (**Figure 2.3A-B**), among which pantothenic acid (B_5) was only increased by 20% algae while pyridoxine (B_6) and riboflavin (B_2) were increased by algae dose-dependently (**Figure 2.5A-C**). To determine whether the increased urinary excretion of B vitamins was correlated with the levels in the diets and inside the body, the distributions of riboflavin and its metabolites in three feeds and the liver were examined. The results showed that algae supplementation elevated the riboflavin level in the feeds dose-dependently, suggesting that the source of increased urinary riboflavin was from the diets (**Figure 2.5D**). In contrast, the levels of riboflavin and its metabolites, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), in the liver were not significantly affected by algae feeding (**Figure 2.5E**), suggesting that extra riboflavin from algae might not been effectively retained inside the body. In addition, prominent changes in color were visible in fecal samples from algae feeding. Two pigments, 3-hydroxy-b,e-caroten-3'-one, a degradation product of carotenoids, and chlorophyllide b, a component of algal chloroplast, were largely deficient in control fecal samples, but present in algae fecal samples dose-dependently (**Figure 2.5F-G**) .

2.3.3.3 Effects of algae feeding on amino acid homeostasis

Algae are considered as an enriched source of protein. Algae powder in this feeding study contains 53% protein (% dry weight), while is much higher than 19% protein in control AIN93G diet. To determine whether the protein inputs from algae-containing diets could affect the homeostasis of amino acids (AAs) in mice, the concentrations of free amino acids (FAAs) in the liver and serum were quantified. The results of quantitative analysis indicated that protein enrichment in algae diets were not translated into the higher levels of FAAs since total concentrations of FAAs in serum and the liver were comparable among three groups of mice (**Table 2.2**). However, selective influences on individual AAs were observed after 4-week algae feeding. In both serum and the liver, taurine, a nonproteinogenic AA, was consistently decreased by algae feeding, while arginine and citrulline, two AAs in urea cycle, were consistently increased by 20% algae (**Table 2.2**). In serum alone, algae feeding was associated with the changes in methionine, glycine, lysine and threonine (**Table 2.2**).

2.3.3.4 Characterization of algae-induced changes in the lipidome

The unique composition of algal lipids, especially the enrichment of polyunsaturated fatty acids (PUFAs), is a major driving force behind the application of algae as dietary supplements. Therefore, algae feeding is expected to affect the lipidome. The PLS-DA model on serum samples indicated that algae feeding affected multiple TAGs, cholesterol

esters (CE), and phospholipids, including phosphatidylcholines (PC), lyso-phosphatidylcholines (LysoPC), and phosphatidylethanolamines (PE) (**Figure 2.3E-F**). These lipid markers were clustered in an HCA-based heat map (**Figure 2.6A**). Analyzing the fatty acid compositions of these markers revealed that 20% algae feeding increased the presence of PUFAs, including α-linolenic acid (18:3), eicosapentaenoic acid (EPA, 20:5), and docosahexaenoic acid (DHA, 20:6), but reduced the distribution of saturated and monounsaturated FAs, including palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), and oleic acid (18:1) in serum lipids (**Figure 2.6A**). More importantly, similar distribution of fatty acids was observed in the heat map of liver lipids (**Figure 2.6B**), suggesting that 20% algae feeding could significantly alter the fatty acid composition inside the body. Besides serum and the liver, algae feeding, especially 20% algae, also increased the levels of fatty acids, including oleic acid, linoleic acid, and α-linolenic acid, in feces (**Figure 2.6C**).

2.3.3.5 Characterization of algae-induced changes in redox balance

Reduced glutathione (GSH) in the liver was positively correlated with 5% algae feeding while oxidized glutathione (GSSG) with 20% algae feeding in the PLS-DA model on hepatic metabolome (**Figure 2.3G-H**). Subsequent quantitative analysis further confirmed that 5% algae feeding significantly increased hepatic GSH level while 20% algae feeding significantly increased hepatic GSSG level (**Figure 2.7A-B**). In addition, three lipid

oxidation products (LOPs), including malondialdehyde (MDA), hexanal, and heptanal, in the liver were increased by algae feeding (**Figure 2.7C**), and another three LOPs, including heptenal, heptadienal, and decadienal in urine were also dose-dependently increased by algae feeding, especially from 20% algae (**Figure 2.7D**). All these observations on thiol antioxidant and LOPs suggested that 20% algae feeding elicited a higher level of oxidative stress while 5% algae feeding was associated with the upregulation of antioxidant system.

2.3.3.6 Characterization of algae-induced changes in intermediate metabolites of nutrient and energy metabolism

Intermediate metabolites are the precursors or metabolites of biologically significant molecules. These metabolites commonly exist as minor components in the metabolome, but can function as the regulators of enzymes and pathways as well as the indicators of metabolic activities. Through examining the metabolites contributing to the groupings in the PLS-DA model of hepatic metabolome, multiple intermediate metabolites associated with algae feeding were identified (**Figure 2.3G-H**). Among these metabolite markers, dephospho-CoA, adenylsuccinic acid, 3'-AMP, and nicotinamide were positively associated with 5% algae feeding (**Figure 2.8A**), while carnitine and acetylcarnitine, glutarylcarniinte, 3'-methylglutarylcarnitine were increased by algae feeding (**Figure 2.8B**).

2.3.3.7 Characterization of algae-induced changes in microbial metabolism

Influences of algae feeding on microbial metabolism were reflected by the metabolite markers contributing to the separation of three mouse groups in the PLS-DA model of fecal and urine metabolomes (**Figure 2.3 A-D**). Short chain fatty acids (SCFAs) and bile acids are two types of metabolites in feces that were dramatically affected by algae. The levels of acetic acid, propionic acid, butyric acid and valeric acid were dose-dependently increased by both 5% and 20% algae feeding (**Figure 2.9A**). Taurine bile salts, including taurocholic acid (TCA), tauromuricholic acid (TMCA), and taurochenodeoxycholic acid (TCDCA) was dramatically and dose-dependently increased by both 5% and 20% algae feeding while secondary bile acids, especially lithocholic acid (LCA) was decreased by 20% algae feeding (**Figure 2.9B**). In urine samples, p-cresol sulfate and p-cresol glucuronide derived for the microbial degradation of tyrosine were decreased by 20% algae feeding (**Figure 2.9C**), while glyco-4-methyl-pentanoic acid and glyco-4-methyl-hexanoic acid, two metabolites that might be derived from bacterial branched fatty acids, were increased by algae feeding (**Figure 2.9D**).

2.4 DISCUSSION

Compared to many plants and animals used as the sources of feed components, algae have different composition of macronutrients, micronutrients, and bioactive compounds (detailed in **1.2**). Therefore, distinctive nutritional, physiological, and metabolic events are

expected after consuming significant amount of algae through feeding. Among these events, the metabolites, either derived from algae directly or produced from algae-elicited metabolic activities, can function as initiators, executors, or end products. In this study, these metabolites were identified and characterized through biochemical and metabolomic analysis. Because the growth-promoting effects of 5% algae and the growth-suppressing efforts of 20% algae on young mice were observed at the end of 4-week algae feedings (**Figure 2.1**), the discussions on these metabolites are largely based on their associations with the dose-dependent effects of algae feeding on growth, as well as their biochemical, physiological, and metabolic functions.

Effects of algae on mouse metabolome. Consumption of foods high in bioactive compounds as supplements is becoming more and more popular. Various health benefits of algae have been proved including lowering-cholesterol, antioxidant and anti-inflammatory effects. However, the lack of evidence for taking *Scenedesmus* algae as supplementation counteracts the use of human consumption (174,175). Most previous studies about the effects of algae on metabolism only reported several metabolites such as LDL cholesterol and lipoprotein lipase, with only a few used *Scenedesmus* as material. Therefore, in this study, the effects of algae feeding on metabolic system were reflected indirectly by the changes in growth performance, also directly by the metabolic changes detected by blood chemistry and metabolomics analysis.

The results of metabolomics analysis of diverse sample including serum, liver, feces and urine not only reconfirmed the hypolipidemic and multivitamins impacts of algae on animals, but also pointed out novel unreported changes in amino acids, redox balance, lipid and microbial metabolism through identification and quantitation of metabolite markers.

Algae are a good source of vitamins, especially water soluble vitamins, including B1, B2, B12 and C. Water soluble vitamins can continuously excreted in the urine of healthy individuals, making deficiency relatively common when the dietary intake is insufficient (176). The significant increased level of riboflavin and pyridoxine in both algae feeding group and pantothenic acid in 20% algae were discovered. Usually, algae have twice amount of riboflavin than RDI recommended for human beings based on the culture condition, therefore, the mice may consume riboflavin in excess of the requirement, and excrete through urine eventually. Pantothenic acid was required by animals since it is a precursor in the synthesis of coenzyme A. The results showed that pantothenic acid level was only increased in 20% algae feeding. In relation, dephospho-CoA, an intermediate compound in CoA biosynthesis, was decreased in 20% feeding. The adverse changes of pantothenic acid and dephospho-CoA may suggest that the pathway of CoA synthesis for mice under 20% algae feeding was inhibited somehow, less pantothenic was needed for this pathway and excessive amount of it was excreted by urine. Further studies are required to confirm and determine the underlying mechanism.

Several interested features were found in 20% algae feeding among redox balance system. 1) The essential AA methionine was increased while taurine was decreased. Methionine is

a sulfur-containing amino acid, and its metabolism including the activation to S-adenosylmethionine and methylation. Homocysteine is one of the products, and it can be remethylated to methionine or converted to cysteine, further, cysteine can be converted to taurine and glutathione. The imbalance concentrations between methionine and taurine suggested algae feeding may affect the transsulfuration pathway. 2) GSH is not an essential nutrient for human and animals since it can be biosynthesized from cysteine, glutamic acid, and glycine. Glutamic was decreased while glycine was increased. However, the reaction of gamma-glutamylcysteine synthesized from glutamic acid and cysteine is the rate-limiting step in glutathione synthesis, which leads to a decreased level in GSH concentration. 3) GSSG was slightly increased, by calculating the ratio between GSH and GSSG, it significantly increased in 5% algae feeding but decreased in 20%. 4) Markers related to the oxidative stress including MDA, heptanal, hexanal, and pentanal have been found increased only in 20% algae feeding group. All those observations suggested that mice fed with 20% algae were under a higher oxidative stresslevel. *Scenedesmus* algae were selected for its high PUFA content, especially high EPA and DHA level, which is essential for proper growth and body function, and must be obtained from the diet since animals cannot synthesize it (177,178). However, those ω-3 PUFA are highly unsaturated and easy to peroxidation (179). Peroxidation will increase the lipid peroxides including MDA and aldehyde breakdown products, decreased glutathione in liver, which have been implicated in disease states such as kidney damage, cardiovascular disease, and others (180). It has been shown that the degree of DHA incorporation in diet can affect the extent of lipid peroxidation, LDL and erythrocyte susceptibility to lipid peroxidation (181).

Therefore, further mechanistic studies are required to examine the validity of whether PUFA in algae can lead to a higher oxidative stress.

PCs and PEs are major phospholipids of plasma membrane. The observed changes in hepatic and serum phospholipids implicated that algae could alter plasma membrane through changing its composition, which has been reported that those alterations may induce the change of membrane fluidity, microviscosity, further influencing the membrane bound enzymes and receptors. TAG is also a key factor in determining coronary artery disease; the changes of TAGs composition induced by algae were also analyzed. EPA and DHA-containing TAGs are dramatically increased in 20% algae feeding, as well as decreased palmitic acid and oleic acid-containing TAGs. Studies showed ω-3 PUFAs are mainly metabolized in hepatic cells by desaturation and elongation of essential fatty acids, and it is proved that ω-3 PUFA levels in plasma may related to those in diets (182). Therefore, the increased level of TGs containing EPA and DHA fatty acids may accounted for the high percentages of these fatty acids in algae diet. Given the fact that phospholipid and TAG are largely determining the chemical and physical properties of biological membranes and membrane enzyme proteins, algae induced changes in lipid metabolism should consider its importance, and further studies are required to determine the underlying mechanism (183).

Besides observing altered fatty acid composition in phospholipids, other metabolites from liver also indicated some changes in lipid metabolism. Carnitine is an important metabolism intermediate in lipid metabolism, which transports long-chain acyl groups

from fatty acids into the mitochondrial, so they can be broken down and produce energy through β-oxidation. As shown in the results, the hepatic level of carnitine and its related intermediates associated with energy production and lipid metabolism through β-oxidation were increased in 20% algae feeding. Animals can either produced carnitine by themselves in both liver and kidney from lysine and methionine or get from diets. Algae have not been reported for high carnitine level, so the changes in liver must happen inside of the animals. Also, nicotinamide, a precursor of NAD, was decreased in 20% feeding but increased in 5% feeding. Those changes indicated that the energy production through β-oxidation decreased in higher dose algae feeding, which may contribute to the energy dysregulation. Further studies may focus on the NAD-related metabolism to confirm these findings on lipid metabolism.

The influence of algae on intestinal function and microbial metabolism were reflected by fecal metabolites. For example, the significant increased level of SCFAs including acetic acid, propionic acid, butyric acid and valeric acid. Since SCFAs originate from microbial fermentation, this observation may suggest an elevated colonic fermentation due to the high content of PUFA in diet (184). Studies have been shown that the dietary fatty acids may involve altering the fatty acid composition of the intestinal wall and thereby modify the attachment site to either promote or inhibit microbial colonization (185). Other possibilities can be the non-digestible carbohydrate contents of algae.

Values and challenges of metabolomics. Since metabolism is a determining factor in growth and wellbeing, metabolomic analysis in this study provided an opportunity to

characterize algae-induced metabolic events and to understand their roles in the growth and wellbeing of algae-fed animals.

Previous investigation on the metabolic effects of algae in humans and animals were mainly based on traditional studies including blood chemistry (186). Targeted analyses of specific metabolites have not been widely used, although this method is incapable of defining global profile of metabolic system or identifying unexpected metabolic event. However, untargeted metabolomics analysis can address the limitation from targeted analyses, providing details on amino acids, lipids, and microbial metabolites induced by algae feeding. The normal procedures of LC-MS-based untargeted metabolomics platform includes sample preparation, LC-MS analysis, multivariate data analysis, marker identification and characterization, therefore, further studies can focus on bioinformatics, pathway analysis and quantitation (137). In this study, the values and challenges of individual metabolomics analysis procedures based on discovering novel metabolite markers are evident and can be summarized as follows. 1) In sample preparation, besides adopting respective extraction methods for each type of samples, chemical derivatization, using DC and HQ, had greatly extended the coverage of LC-MS analysis and facilitated the quantitation of interested metabolites in this study. Considering chemical derivatization is a requisite step in GC-MS-based metabolomics, the application of chemical derivatization in LC-MS-based metabolomics remains to be limited and can be greatly expanded. 2). PCA and HCA, two multivariate methods, were conducted to process complex LC-MS data in this study. PCA modeling was able to show diet-associated

grouping of examine samples, based on sample distribution in the score plots . Subsequently, the interested metabolite markers identified by PCA were processed by HCA, which produced the heat maps revealing both the correlations among multiple as well as dose-dependent changes**.** This combination of PCA and HCA facilitated data visualization and marker identification, and is an efficient approach for examining complex metabolomics datasets. 3) Despite the progresses in constructing metabolomic databases and bioinformatics tools, marker identification remains to be a bottleneck in many metabolomics efforts. In this study, multiple candidate structures with the same elemental composition were commonly enlisted for interested metabolites by accurate mass-based database search, which reduced the choices for further structural elucidation. However, unambiguous structural identification of these metabolite markers was still achieved by the comparison of authentic standards or the analysis of MSMS fragmentograms, which in many cases corrected the structure proposed by initial analysis. Since many recent published metabolomics works heavily relied on bioinformatics-based structural identification, the experience in this study highlights the need to be cautious when processing the results from database search.

2.5 CONCLUSION

In summary, compared to limited coverage of blood chemistry analysis, the comprehensive metabolomics analysis in this study provided many novel information on algae-induced

metabolic events. Feeding 20% algae decreased the cholesterol level and increased the levels of B vitamins and PUFAs, but these metabolic effects were not correlated with the improvement in growth performance. In contrast, the status of redox system appears to be better correlated with growth performance and health status. In addition, algae feeding affected specific intermediate metabolites in nutrient and energy metabolism, and disrupted microbial metabolism. Overall, the observed correlations between the metabolites and growth performance in algae feeding could serve as a foundation for establishing biomarkers of algae-induced biological effects or conducting further mechanistic investigations to guide the uses of algae as dietary supplements in humans and feed components in animals.

Table 2.1 Effects of algae feeding on hepatic, serum, fecal and urinary metabolomes. The metabolite markers of each diet were identified through their positive correlations with the diet in the PLS-DA models of LC-MS data.

	Markers of control diet	Markers of 5% algae diet	Markers of 20% algae diet
Feces	Lithocholic acid, coprocholic acid, hydrocinnamic acid, Eicosatrienoic acid, 3-hydroxy-hexadecanoic acid, 12-ketodeoxycholic acid, 2-Indolecarboxylic acid.	N-acetylhistamine, PE(16:0/0:0), pyroglutamic acid, muricholic acid, deoxycholic acid	Alpha-linolenic acid, linoleic acid, oleic acid, tauromuricholic acid, taurochenodeoxycholic acid, acetic acid, butyric acid, propinoic acid, valeric acid, 3-hydroxy-b,e-caroten-3'-one, chlorophyllide b.
Serum	Taurine, threonine, glutamate, lysine, palmitoleic acid, PC(16:0/22:6), PC(16:0/22:4), PC(20:4/22:6), PC(18:0/22:6), PC(18:0/20:3), PC(18:1/20:3), LysoPC(18:1), LysoPC(18:2), LysoPC(18:1), LysoPC(20:3), LysoPC(22:6), TG(16:0/16:0/18:1), TG(16:0,18:1/20:1), TG(16:1/16:1/18:1), TG(16:1/18:1/18:1), TG(18:1/18:2/20:1), TG(18:1/18:1/20:1), TG(18:1/18:1/18:1), TG(18:1/18:1/18:2)	Aspartic acid, suberic acid, sebacic acid, PC(16:0/22:6), PC(18:1/20:4), PC(18:2/20:3), LysoPC(20:4)	Methionine, hydroxyproline, glycine, arginine, sebacic acid, capric acid, CE(20:5), LysoPC(18:3), LysoPC(20:5), PE(16:0/20:0), PC(16:0/20:5), PC(16:0/18:2), PC(15:0/18:2), PC(20:0/20:4), PC(18:0/18:2), PC(18:0,20:2), PC(18:3/22:6), TG(18:2/18:2/18:3), TG(20:4/18:1/20:5), TG(20:5/18:1/22:6), TG(20:5/18:1/22:6), TG(20:4/18:1/20:5)

Liver	Taurine, arginine, guanosine monophosphate, LysoPC(16:0), adenine	Glutathione, aspartic acid, dephospho-CoA, adenylsuccinic acid, 3'-AMP, LysoPC(20:4), LysoPC(18:2), LysoPC(18:3), glutamic acid, niacinamide, hypoxanthine	Oxidized glutathione, citrulline, PC(16:0/16:0), PC(18:0/22:6), PC(18:0/18:2), PE(18:0/22:6), PC(18:0/22:6), PC(16:0/22:4), PC(16:0/20:5), PC(16:2/18:3), PE(18:0/20:4), PE(18:1/20:1), PE(16:0/22:6), L-carnitine, acetylcarnitine, 3-dehydroxycarnitine, glycerylphophorylethanolamine,3-methylglutarylcarnitine, 5-methylthioadenosine, MDA, heptanal, hexanal, pentanal,
Urine	p-cresol sulfate, p-cresol glucuronide, glycine, indolelactic acid, 3-oxo-4-pentenoic acid, phenylalanine	Ribothymidine	Riboflavin, pyridoxine, pantothenic acid, glycol-conjugated pentanoic acid, glycol-conjugated hexanoic acid, heptenal, decadienal, heptadienal, sebacic acid, capric acid, hippuric acid

Table 2.2 Concentrations of free amino acids in serum and liver. Values are means ± SD. Significant differences between groups are determined by the Bonferroni *t*-test. Different superscripts (^{a} or ^{b}) indicate significance differences.

	Serum (μM)			Liver (μg/g tissue)		
	CTL	5%	20%	CTL	5%	20%
Ala	209.35	236.3	235.01	231.88	237.36	203.08
Val*	242.94	276.93	270.08	58.58	59.92	49.52
Ile*	120.65	139.01	151.53	37.01	33.53	37.99
Phe*	89.85	99.04	108.95	3.94	3.28	7.58
Tyr	121.85	140.49	153	59.2	65.52	58.84
Try*	133.69	142.41	153.9	18.08	17.37	17.1
Met*	25.54^{a}	35.83^{ab}	41.96^{b}	391.9	443.58	317.37
Tau	774.51^{a}	698.4^{a}	564.45^{b}	2844.17^{a}	2682.88^{b}	2358.5^{b}
Pro	125.51	154.58	164.88	27.47	26.11	27.2
Asn	59.06	63.24	66.88	15.82	14.93	15.03
Asp	16.18^{ab}	19.06^{b}	14.06^{a}	17.69	28.41	16.76
Gln	624.04	612.6	655.91	1025.03	990.45	976.34
Glu	88.73	101.84	76.75	367.99	419.91	316.26
Gly	302.98^{a}	325.65^{ab}	383.4^{b}	298.89	273.62	249.56
Ser	198.01	210.75	209.04	26.43	21.61	31.01
Thr*	199.59^{a}	207.78^{ab}	203.46^{b}	29.23	27.15	25.39
Lys*	649.53^{a}	605.36^{b}	698.1^{b}	100.18	88.21	94.53
Arg	189.76^{ab}	169.36^{a}	251.15^{b}	4.42^{a}	4.58^{ab}	4.94^{b}
His	97.93	104.71	97.46	102.56	101.49	93.93
Cit	63.85^{ab}	59.09^{a}	87.03^{b}	5.56^{a}	4.02^{a}	9.31^{b}
Total	4437.7	4496.8	4602.9	5636.03	5522.09	4878.05

*: essential amino acids

Figure 2.1 Influences of algae feeding on growth performance. The body weight and feed intake of three groups of mice (control, 5% algae, 20% algae) were monitored during the 28-day feeding period. ***A.*** Average daily gain (ADG). ***B.*** Average daily food intake (ADFI). ***C.*** Feeding efficiency (G:F ratio). Values are means ± SD (n=8). Significant differences between groups are determined by the Bonferroni *t*-test and labeled as *($P < 0.05$) and **($P < 0.01$).

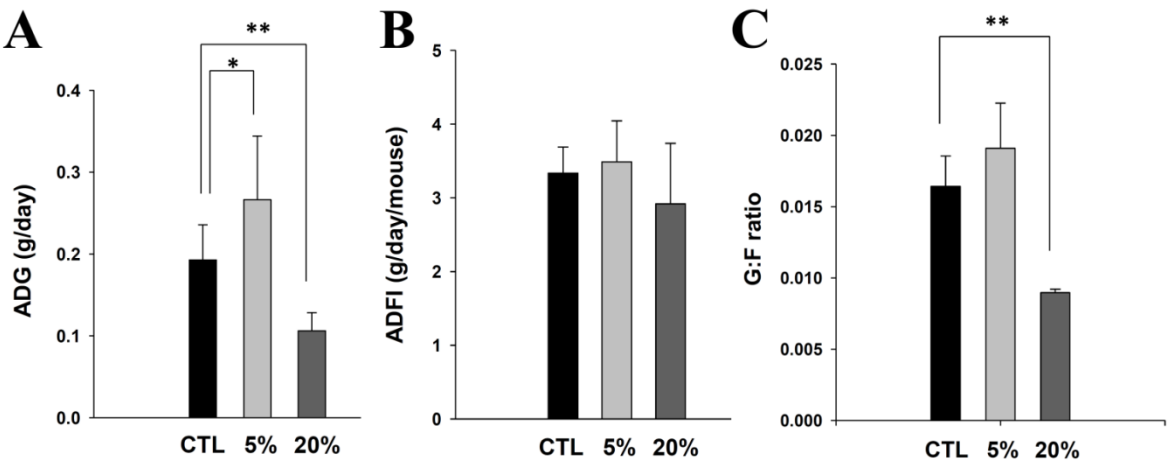
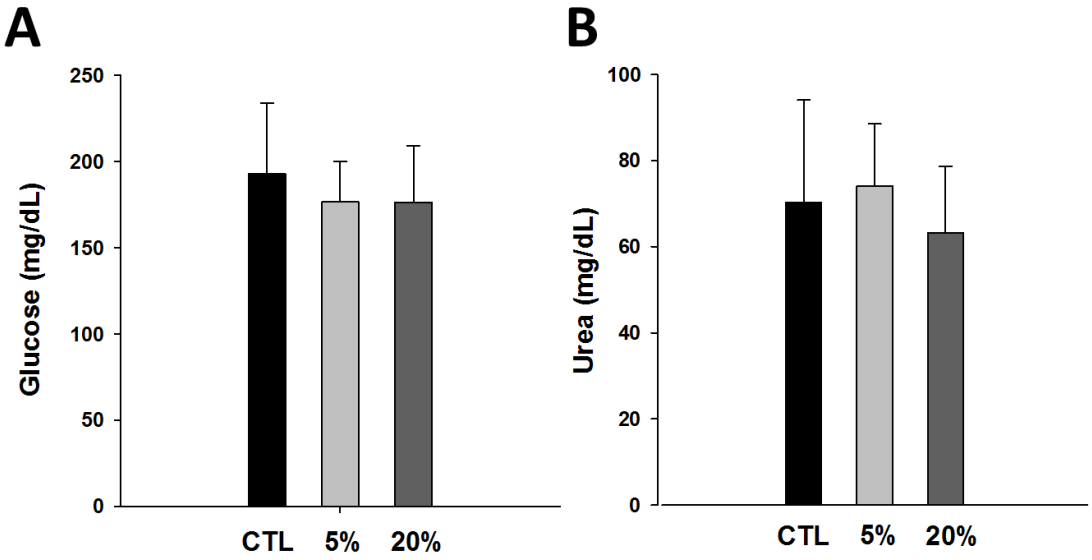
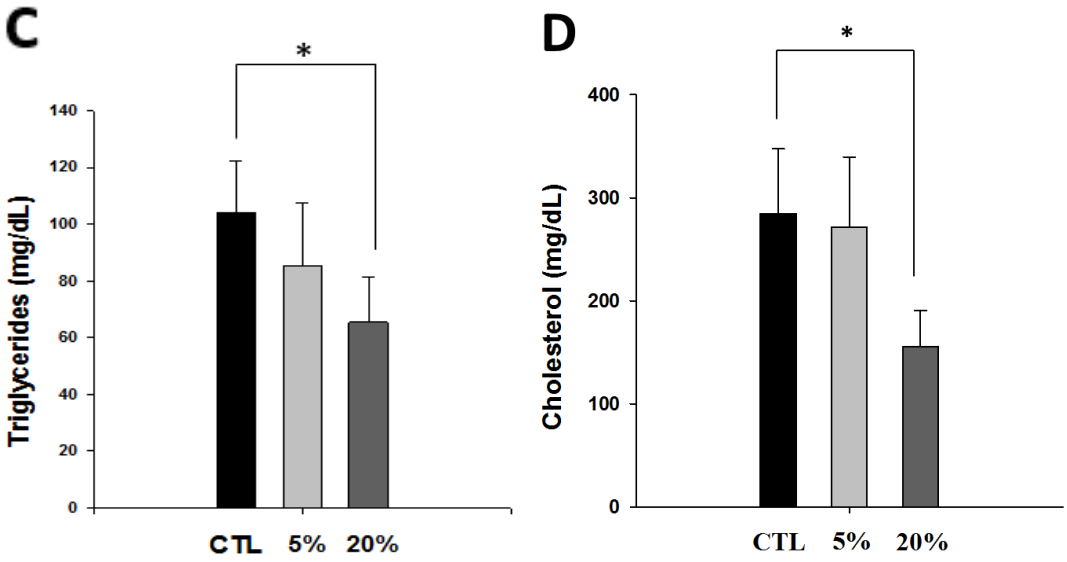


Figure 2.2 Influences of algae feeding on blood biochemicals. ***A.*** Serum glucose level. ***B.*** Serum BUN level. ***C.*** Serum TAG level. ***D.*** Serum cholesterol level. ***E.*** Serum ALT activity. ***F.*** Serum AST activity. Values are means ± SD (n=8). Significant differences between groups are determined by the Bonferroni *t*-test and labeled as *($P < 0.05$) and **($P < 0.01$).




C
*
Triglycerides (mg/dL)
140
120
100
80
60
40
20
0
CTL
5%
20%
D
*
Cholesterol (mg/dL)
400
300
200
100
0
CTL
5%
20%

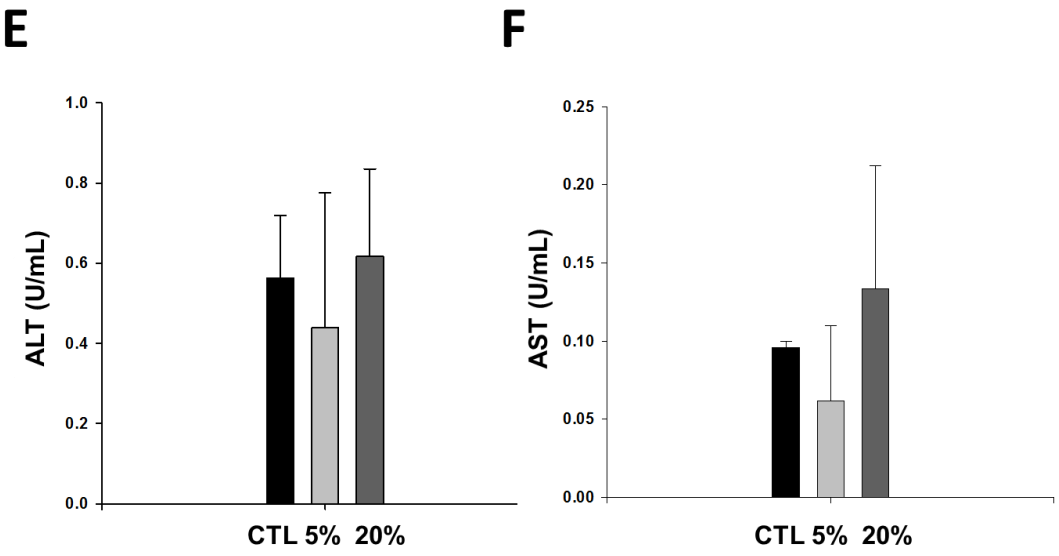
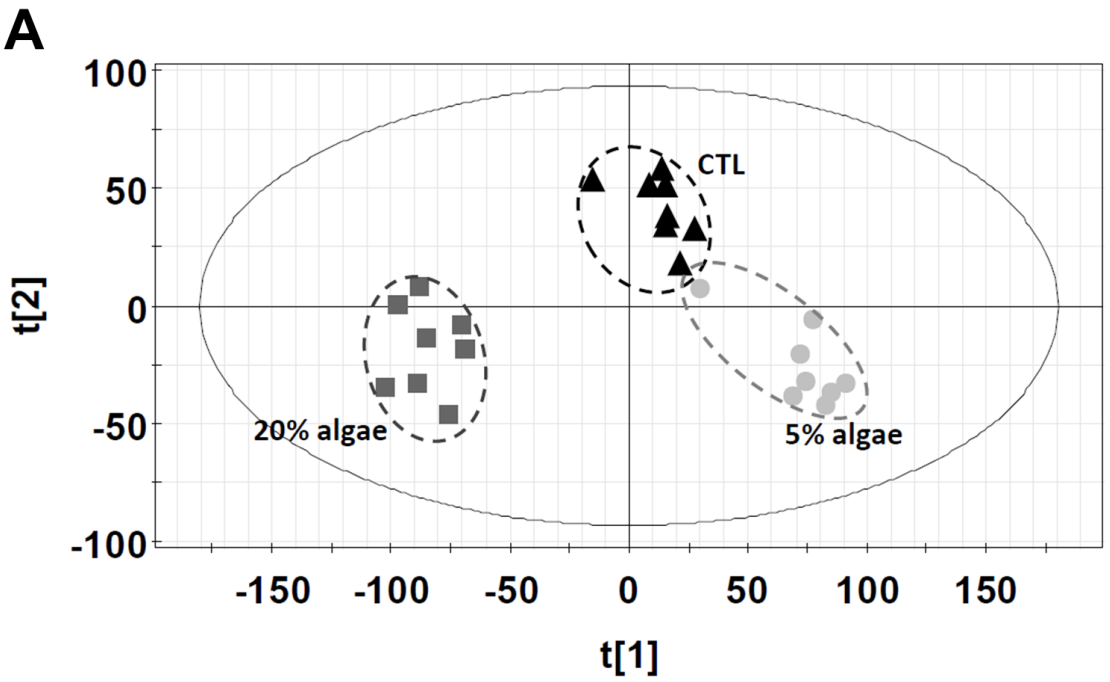
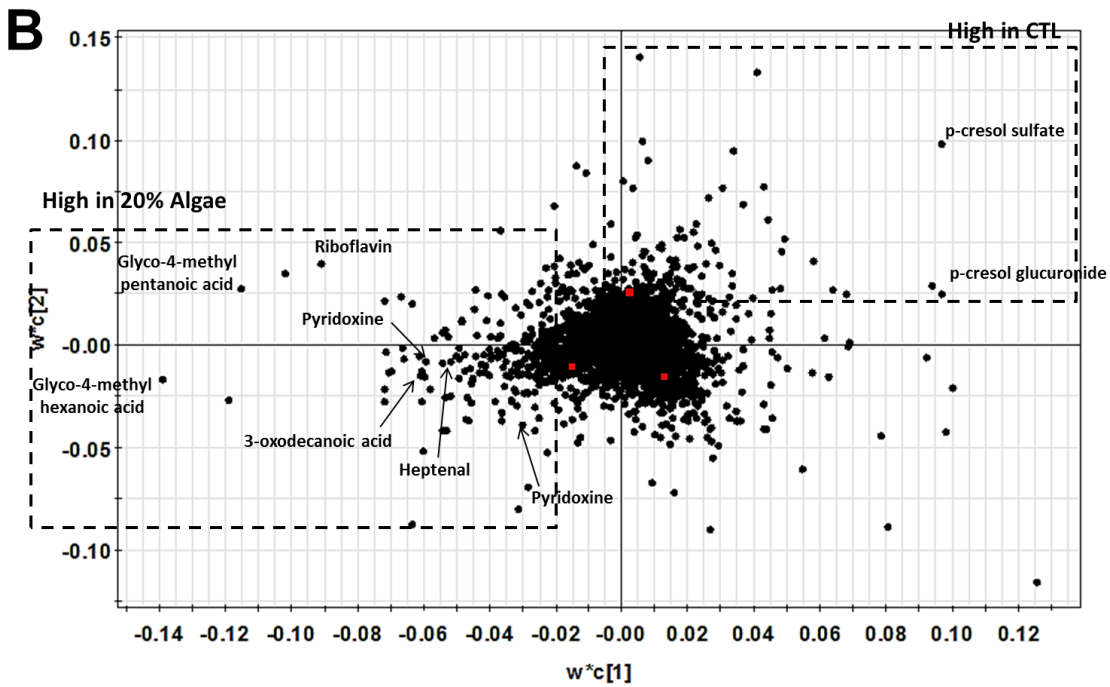
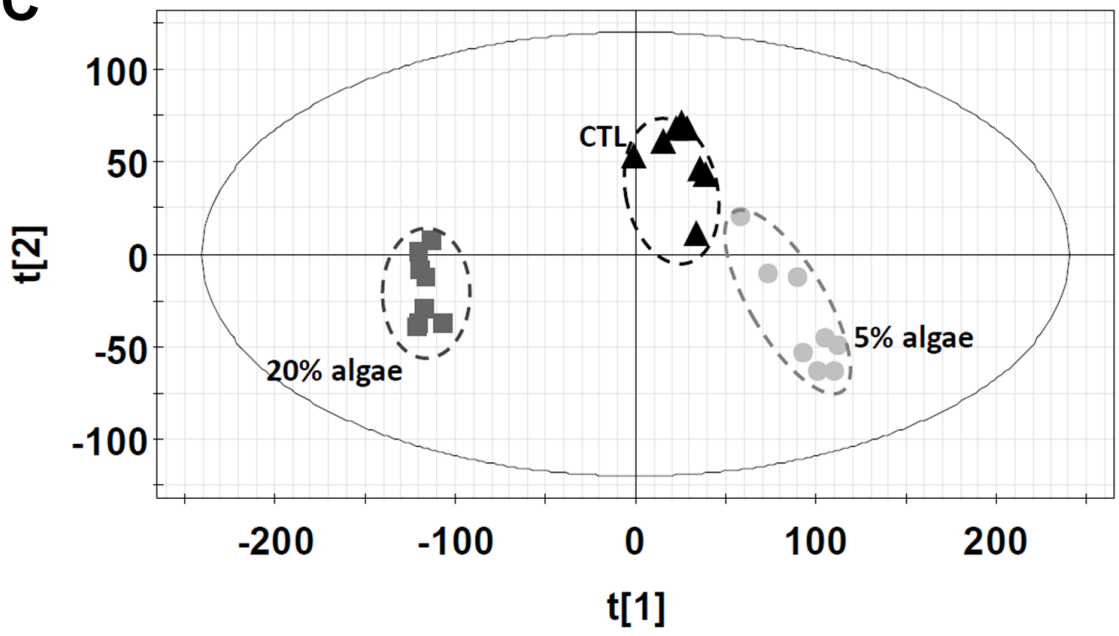

E
F
1.0
0.8
0.6
0.4
0.2
0.0
ALT (U/mL)
CTL 5% 20%
0.25
0.20
0.15
0.10
0.05
0.00
AST (U/mL)
CTL 5% 20%

Figure 2.3 Effects of algae feeding on urinary, fecal, serum, and hepatic metabolomes. Data from LC-MS analysis of urine, fecal extract, serum, and liver extracts were processed by supervised PLS-DA modeling. The relations among three groups of mice (n=8/group) are shown in the Scores plots. The metabolite markers correlating to the diets are labeled in the loadings plots. ***A.*** Scores plot of a PLS-DA model on urine samples. ***B.*** Loadings plot of a PLS-DA model on urine samples. ***C.*** Scores plot of a PLS-DA model on fecal samples. ***D.*** Loadings plot of a PLS-DA model on fecal samples. ***E.*** Scores plot of a PLS-DA model on serum samples. ***F.*** Loadings plot of a PLS-DA model on serum samples. ***G.*** Scores plot of a PLS-DA model on liver extracts. ***H.*** Loadings plot of a PLS-DA model on liver extracts.

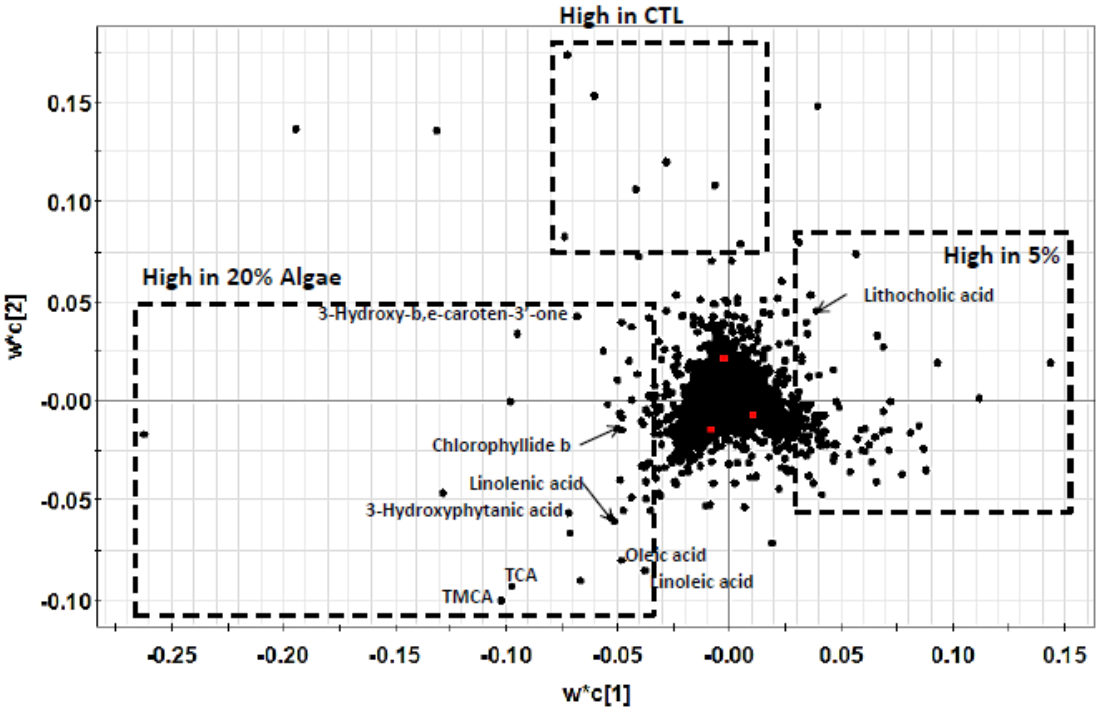

A
100
50
0
-50
-100
t[2]
CTL
20% algae
5% algae
-150
-100
-50
0
50
100
150
t[1]


B
High in CTL
p-cresol sulfate
High in 20% Algae
Riboflavin
Glyco-4-methyl pentanoic acid
p-cresol glucuronide
Pyridoxine
Glyco-4-methyl hexanoic acid
3-oxodecanoic acid
Heptenal
Pyridoxine
0.15
0.10
0.05
-0.00
-0.05
-0.10
w*c[2]
-0.14
-0.12
-0.10
-0.08
-0.06
-0.04
-0.02
-0.00
0.02
0.04
0.06
0.08
0.10
0.12
w*c[1]

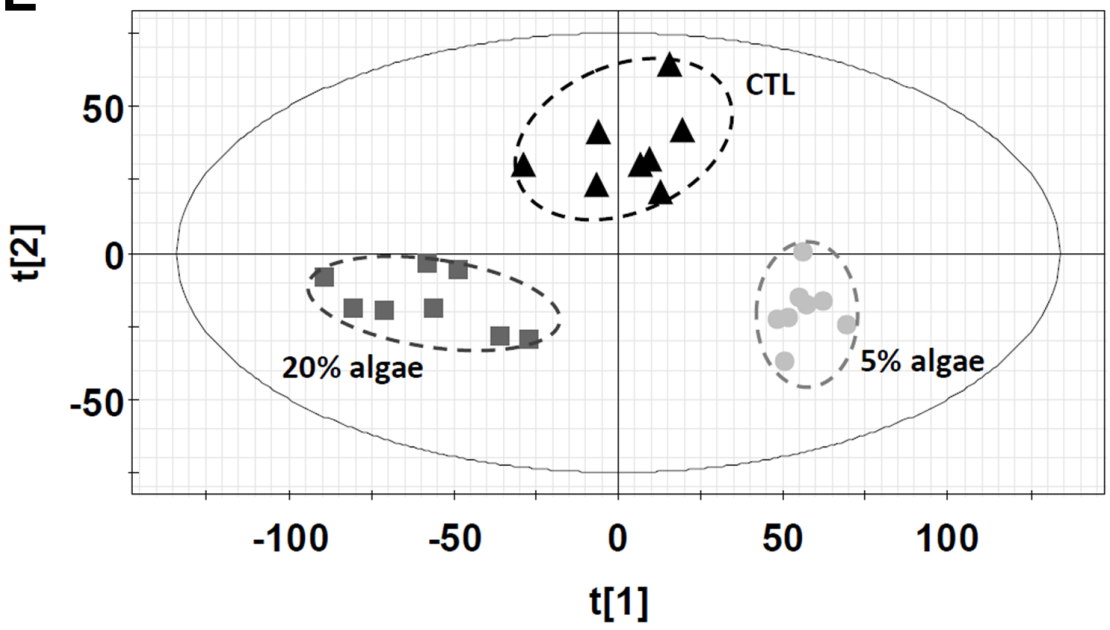
C


100
50
0
-50
-100
t[2]
CTL
20% algae
5% algae
-200
-100
0
100
200
t[1]

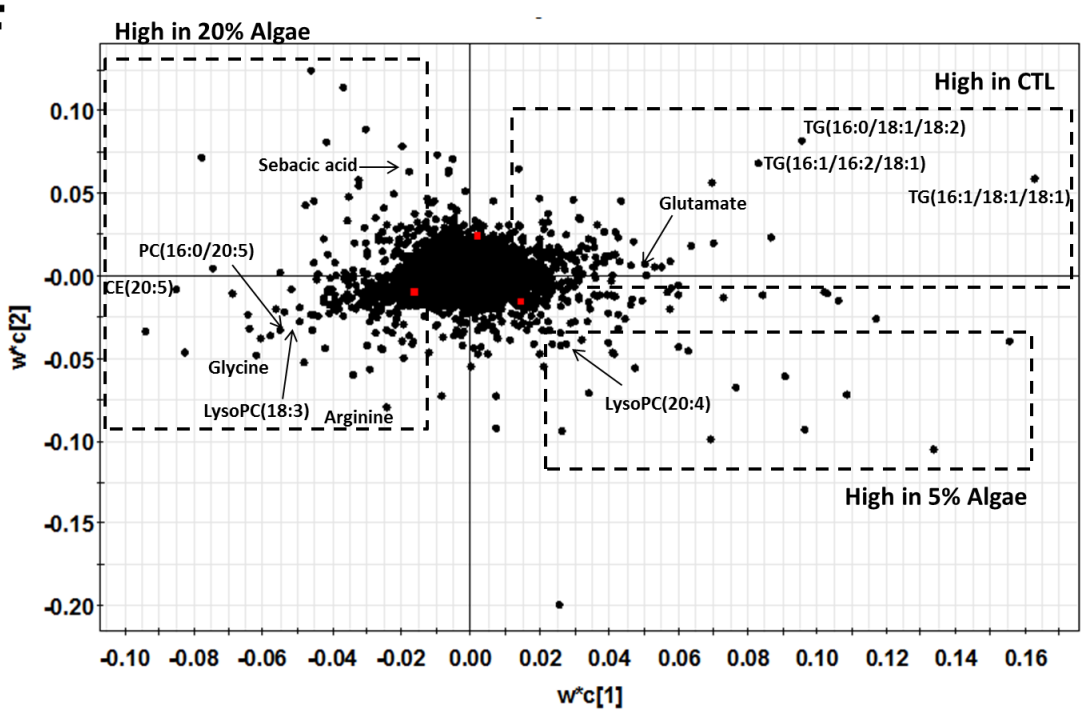
D


High in CTL
High in 20% Algae
High in 5%
3-Hydroxy-b,e-caroten-3'-one
Lithocholic acid
Chlorophyllide b
Linolenic acid
3-Hydroxyphytanic acid
Oleic acid
Linoleic acid
TCA
TMCA
0.15
0.10
0.05
-0.00
-0.05
-0.10
w*c[2]
-0.25
-0.20
-0.15
-0.10
-0.05
-0.00
0.05
0.10
0.15
w*c[1]

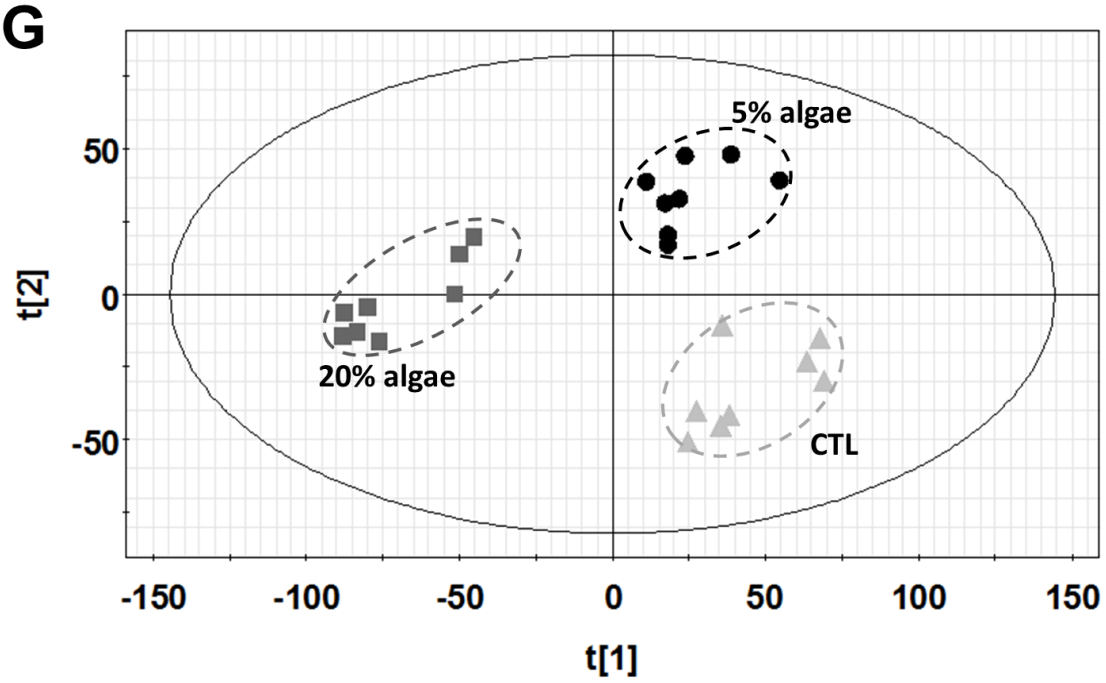
E


CTL
20% algae
5% algae
50
0
-50
t[2]
-100
-50
0
50
100
t[1]

F


High in 20% Algae
High in CTL
High in 5% Algae
Sebacic acid
PC(16:0/20:5)
CE(20:5)
Glycine
LysoPC(18:3)
Arginine
Glutamate
TG(16:0/18:1/18:2)
TG(16:1/16:2/18:1)
TG(16:1/18:1/18:1)
LysoPC(20:4)
w*c[2]
w*c[1]
0.10
0.05
-0.00
-0.05
-0.10
-0.15
-0.20
-0.10
-0.08
-0.06
-0.04
-0.02
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16

G


5% algae
20% algae
CTL
t[1]
t[2]
50
0
-50
-150
-100
-50
0
50
100
150

H

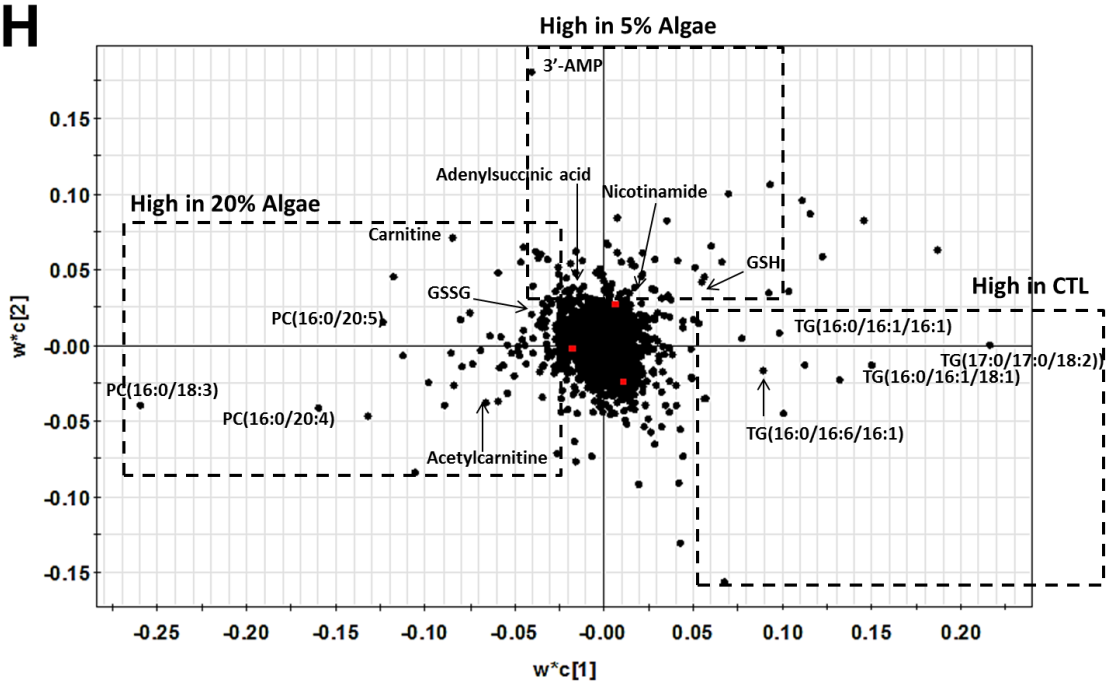
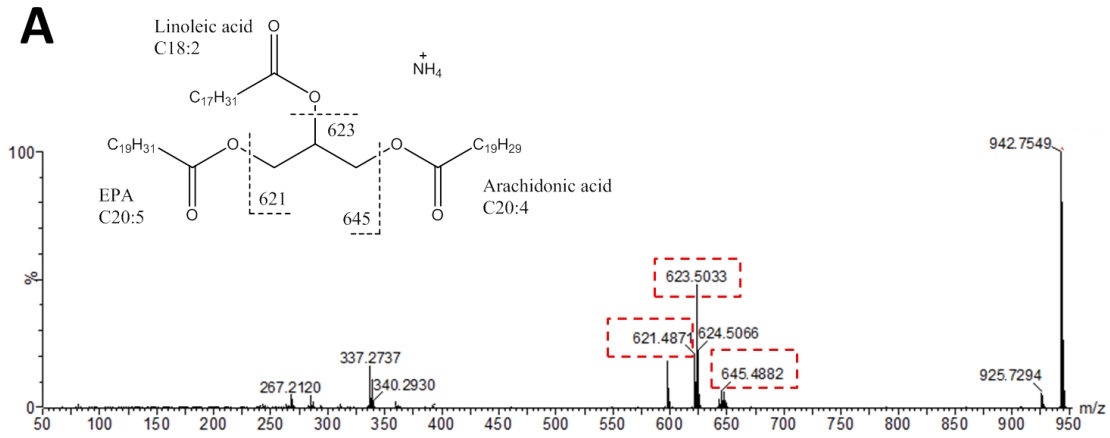
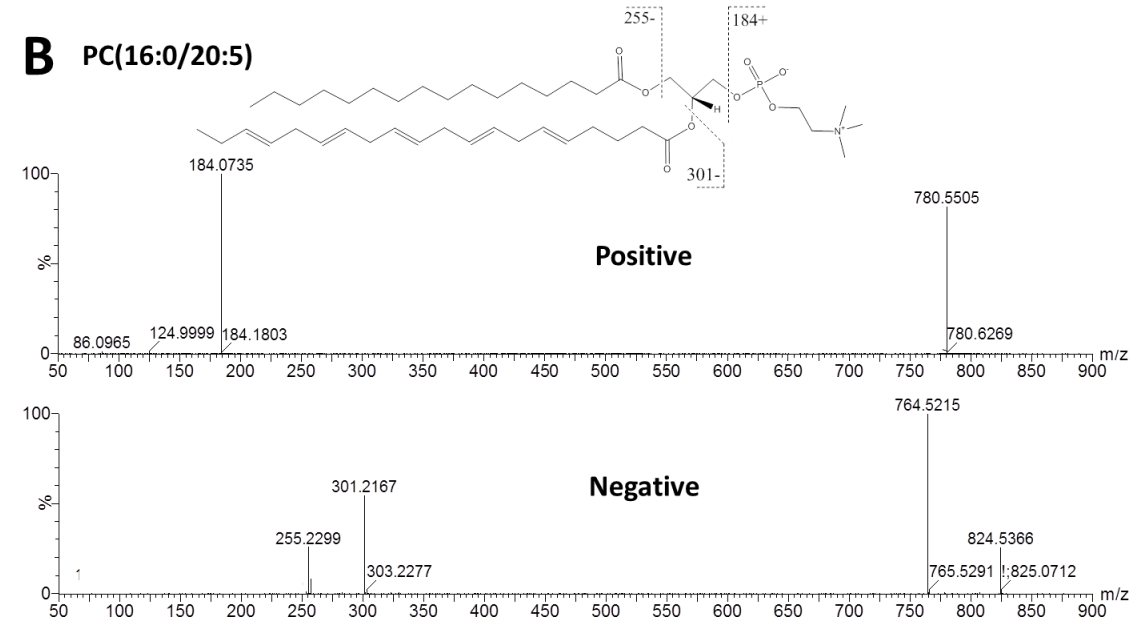
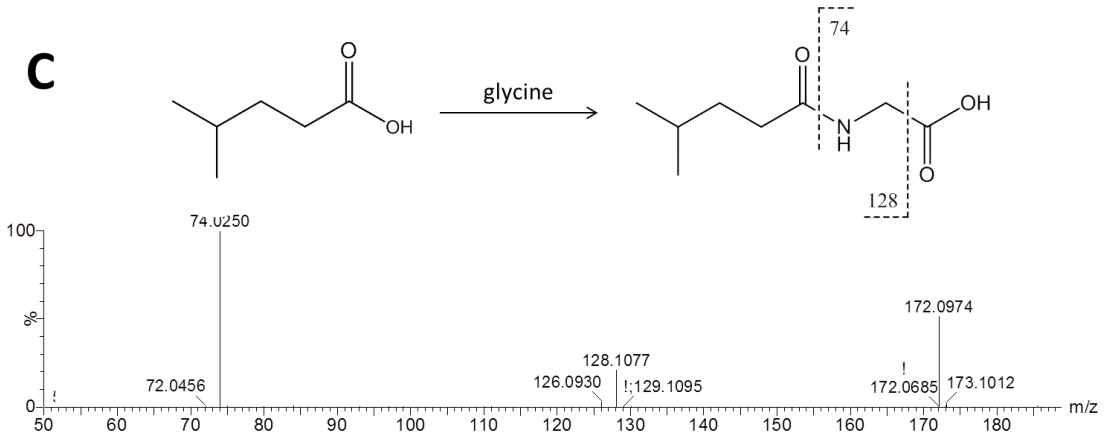

High in 5% Algae
High in 20% Algae
High in CTL
3'-AMP
Adenylsuccinic acid
Nicotinamide
Carnitine
GSH
GSSG
PC(16:0/20:5)
TG(16:0/16:1/16:1)
TG(17:0/17:0/18:2)
TG(16:0/16:1/18:1)
PC(16:0/18:3)
PC(16:0/20:4)
TG(16:0/16:6/16:1)
Acetylcarnitine
w*c[1]
w*c[2]

Figure 2.4 Structural elucidation of metabolite markers. The fatty acid compositions of TAGs and phospholipids were determined by MSMS fragmentation. The glycine conjugates of two branched fatty acids were confirmed by standard synthesis and MSMS fragmentation. ***A***. Structure of TAG(18:2/20:4/20:5) and its MSMS fragmentogram. ***B.*** Structure of PC(16:0/20:5) and its MSMS fragmentograms in both positive and negative ionization modes. ***C.*** Structure of glycol-4-methyl pentanoic acid and its MSMS fragmentogram. ***D.*** Structure of glycol-4-methyl hexanoic acid and its MSMS fragmentogram.


A
Linoleic acid
C18:2
$C_{17}H_{31}$
NH_4^+
623
$C_{19}H_{31}$
$C_{19}H_{29}$
EPA
C20:5
621
645
Arachidonic acid
C20:4
942.7549
623.5033
621.4871
624.5066
645.4882
337.2737
267.2120
340.2930
925.7294
100
%
0
50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950
m/z


B
PC(16:0/20:5)
255-
184+
301-
184.0735
780.5505
Positive
86.0965
124.9999
184.1803
780.6269
100
%
0
50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900
m/z
764.5215
301.2167
Negative
255.2299
824.5366
303.2277
765.5291
825.0712
100
%
0
50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900
m/z

C


O
OH
glycine
74
O
N
H
OH
O
128
100
%
0
74.0250
72.0456
126.0930
128.1077
129.1095
172.0685
172.0974
173.1012
50 60 70 80 90 100 110 120 130 140 150 160 170 180
m/z

D

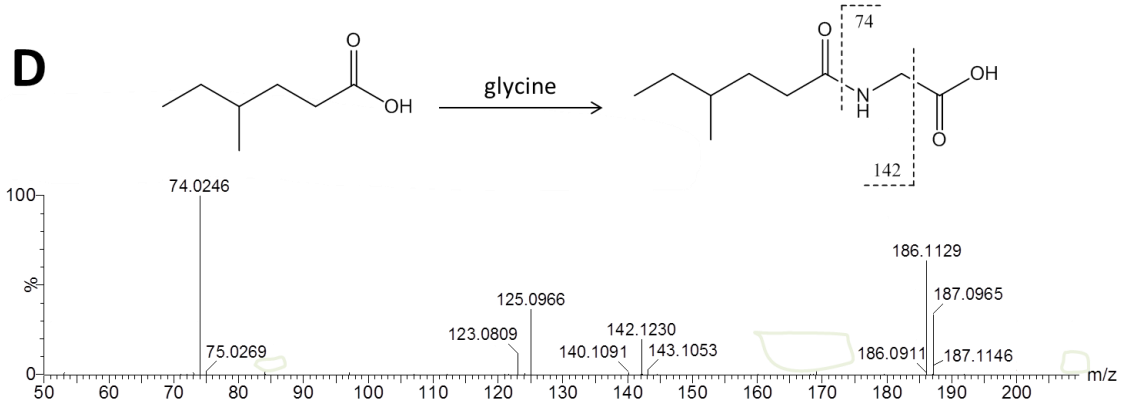
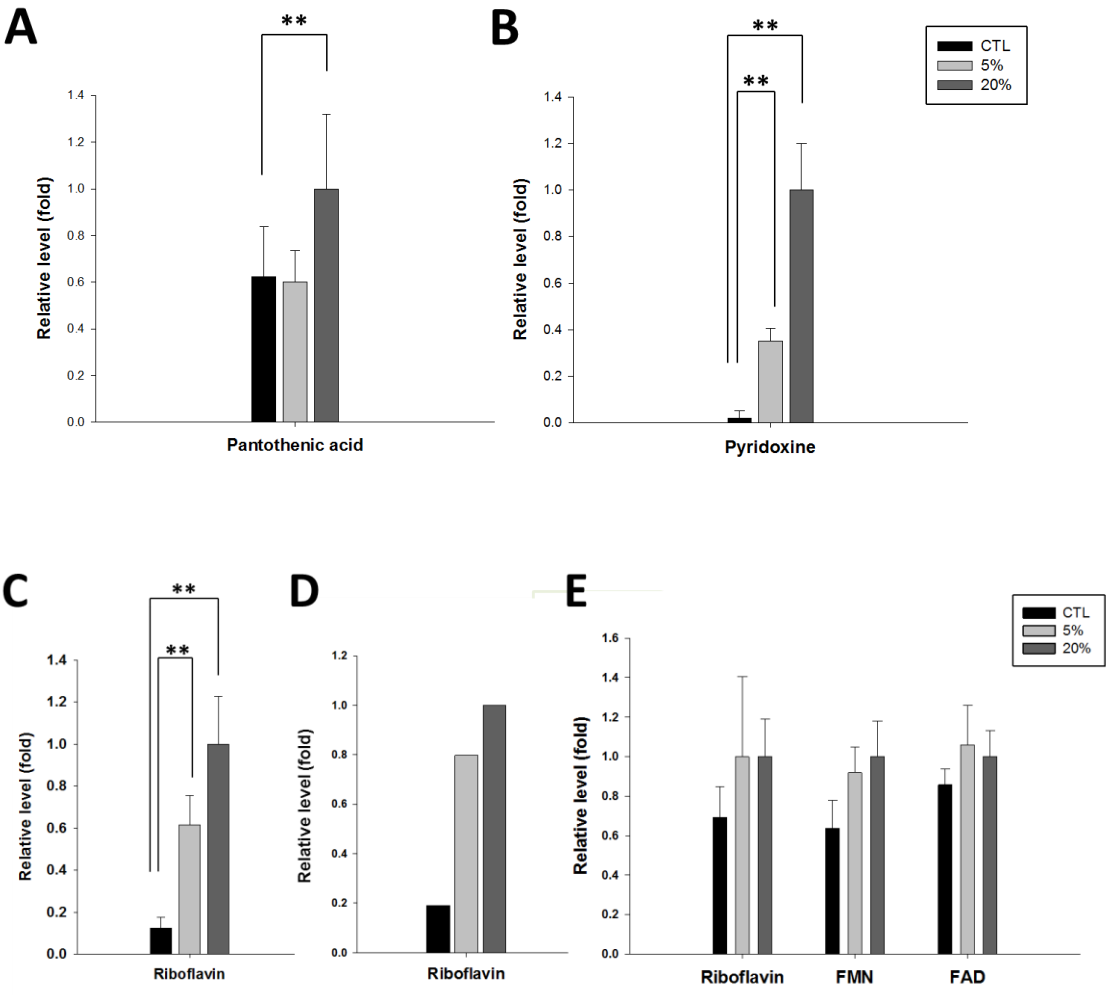

O
OH
glycine
74
O
N
H
OH
O
142
100
%
0
74.0246
75.0269
123.0809
125.0966
140.1091
142.1230
143.1053
186.0911
186.1129
187.0965
187.1146
50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200
m/z

Figure 2.5 Vitamins and pigments as algae exposure markers in urine and feces. The level of each metabolite marker is expressed as the fold of its highest level in three groups of mice or three diets. The highest level is artificially set as 1. ***A.*** Pantothenic acid in urine. ***B.*** Pyridoxine in urine. ***C.*** Riboflavin in urine. ***D.*** Riboflavin in three different feeds. ***E.*** Riboflavin, FMN, and FAD in the liver. ***F***. 3-Hydroxy-b,e-caroten-3'-one in feces. ***G***. Chlorophyllide b in feces. Except the riboflavin in the feeds, values are means ± SD (n=8). Significant differences between groups are determined by the Bonferroni *t*-test and labeled as *($P < 0.05$) and **($P < 0.01$).



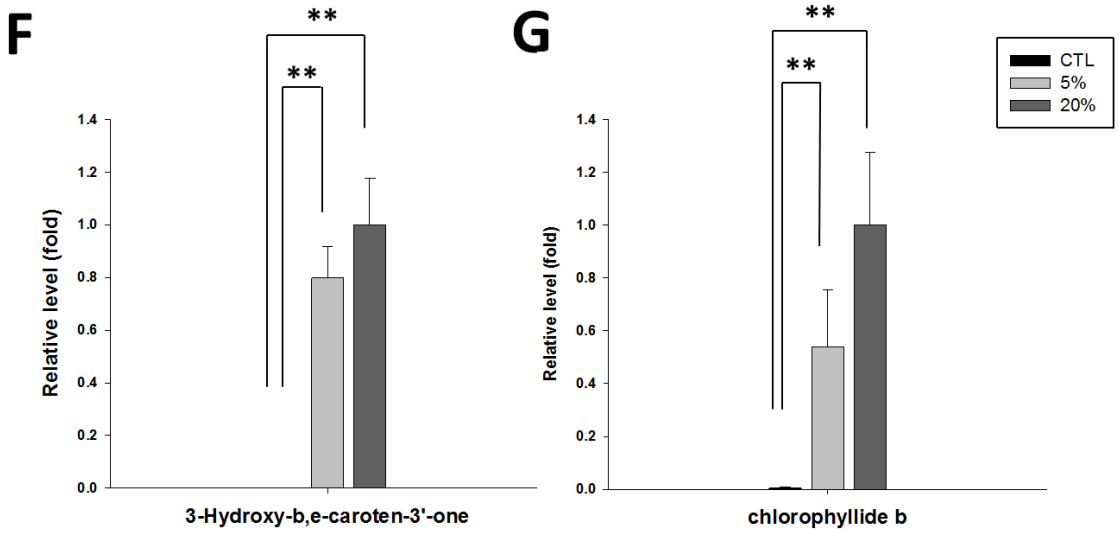
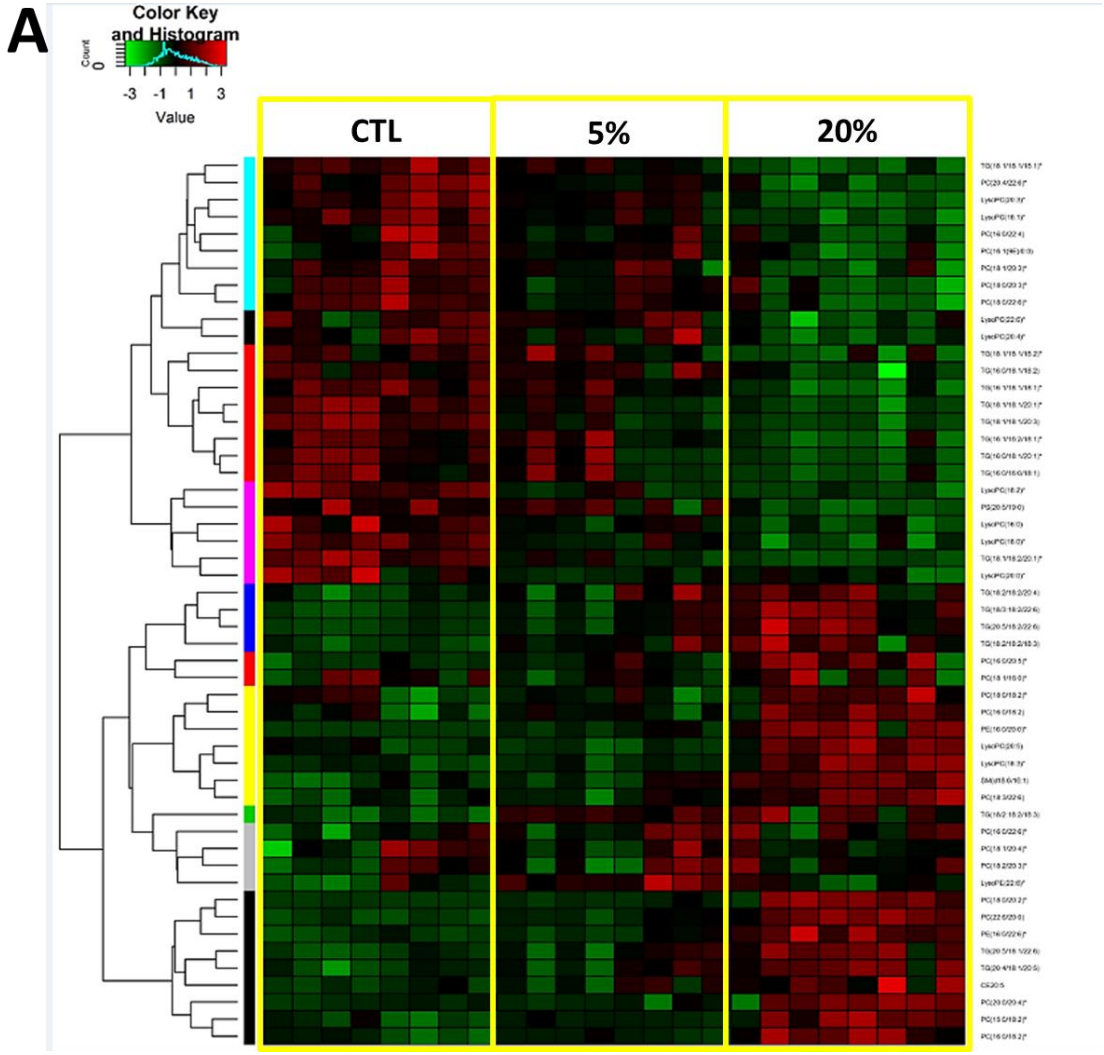
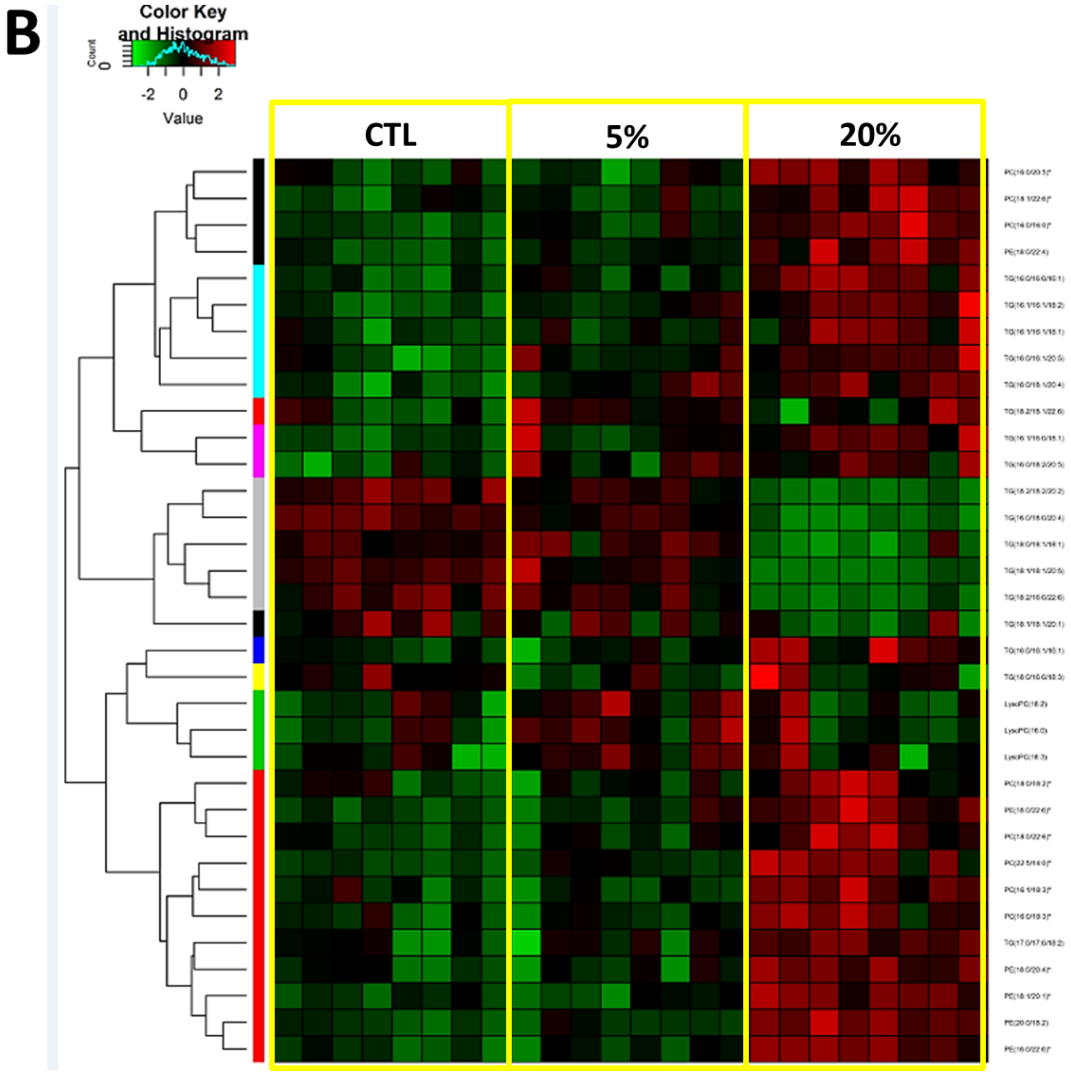

F
**
**
Relative level (fold)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
3-Hydroxy-b,e-caroten-3'-one
G
**
**
Relative level (fold)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
chlorophyllide b
CTL
5%
20%

Figure 2.6 Lipid markers of algae feeding in serum, liver, and feces. *A.* HCA-based heat map on serum lipid markers of algae feeding. The levels of each metabolite markers in total 24 samples are compared by their Z scores and then presented according to inlaid color key. ***B.*** HCA-based heat map on liver lipid markers of algae feeding. ***C.*** Lipid metabolite markers in fecal samples. The level of each metabolite marker is expressed as the fold of its highest level in three groups of mice. The highest level is artificially set as 1. Values are means ± SD (n=8). Significant differences between groups are determined by the Bonferroni *t*-test and labeled as *($P < 0.05$) and **($P < 0.01$).


A
Color Key and Histogram
Count
0
-3
-1
1
3
Value
CTL
5%
20%


B
Color Key and Histogram
Count
0
-2
0
2
Value
CTL
5%
20%

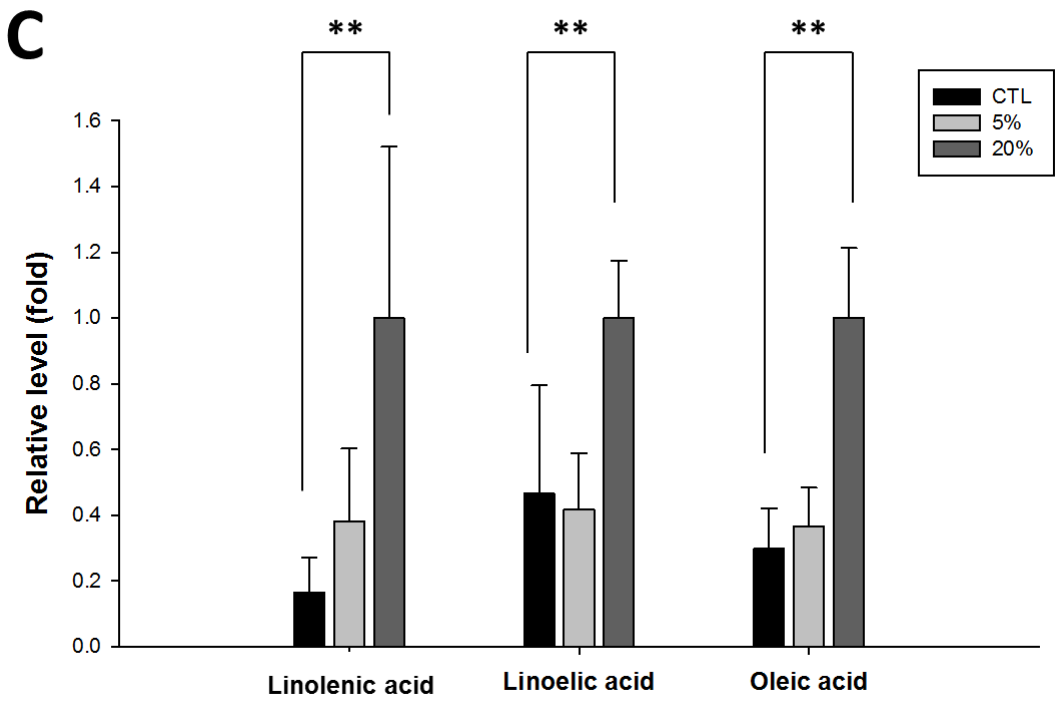

C
**
**
**
CTL
5%
20%
Relative level (fold)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Linolenic acid
Linoelic acid
Oleic acid

Figure 2.7 Effects of algae feeding on hepatic and urinary metabolites associated with redox status. The level of each metabolite marker is expressed as the fold of its highest level in three groups of mice. The highest level is artificially set as 1. **A.** GSH in the liver. ***B.*** GSSG in the liver. ***C.*** Aldehyde LOPs in the liver. ***D.*** Aldehyde LOPs in urine. Values are means ± SD (n=8). Significant differences between groups are determined by the Bonferroni *t*-test and labeled as *($P < 0.05$) and **($P < 0.01$).

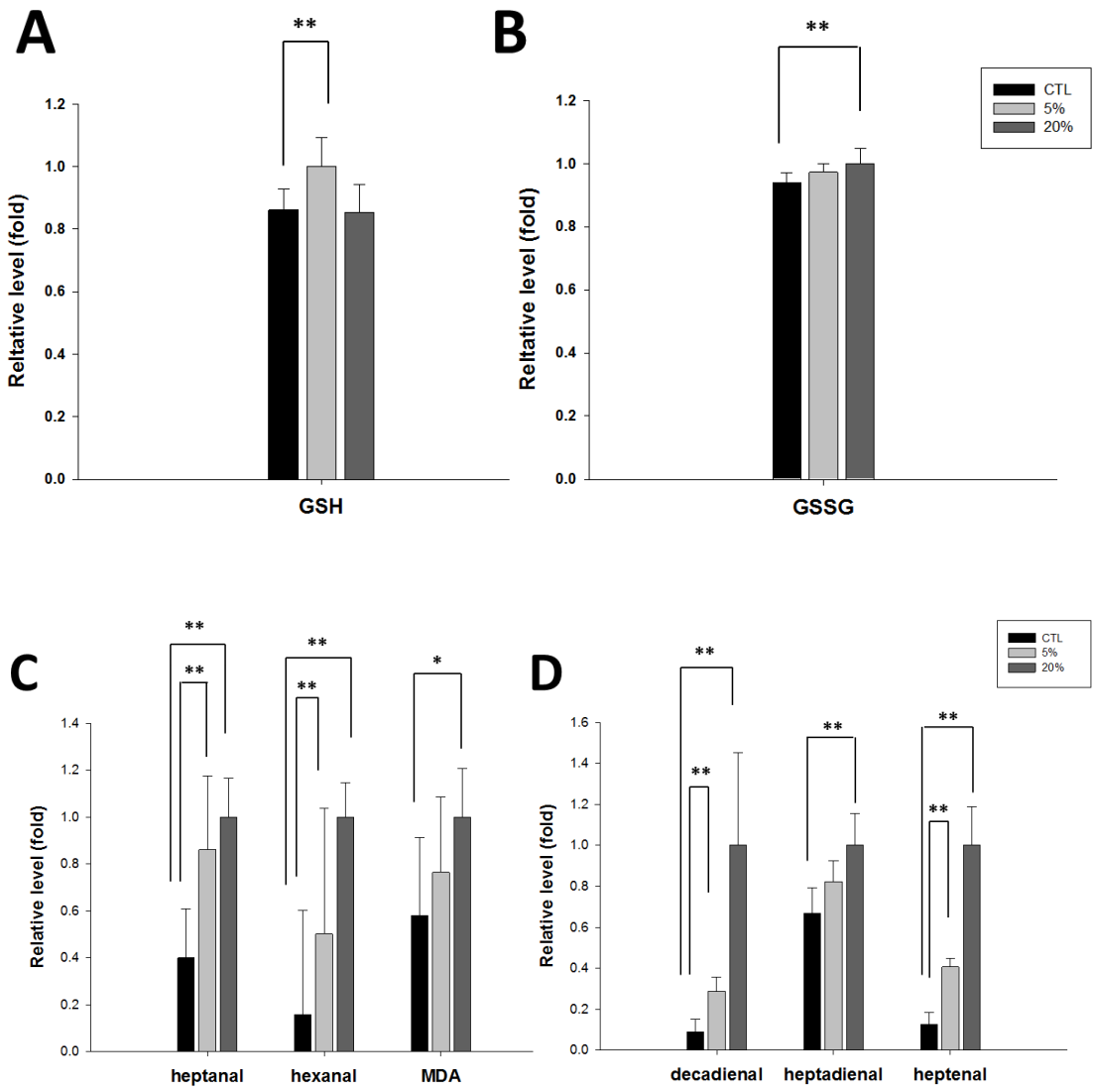
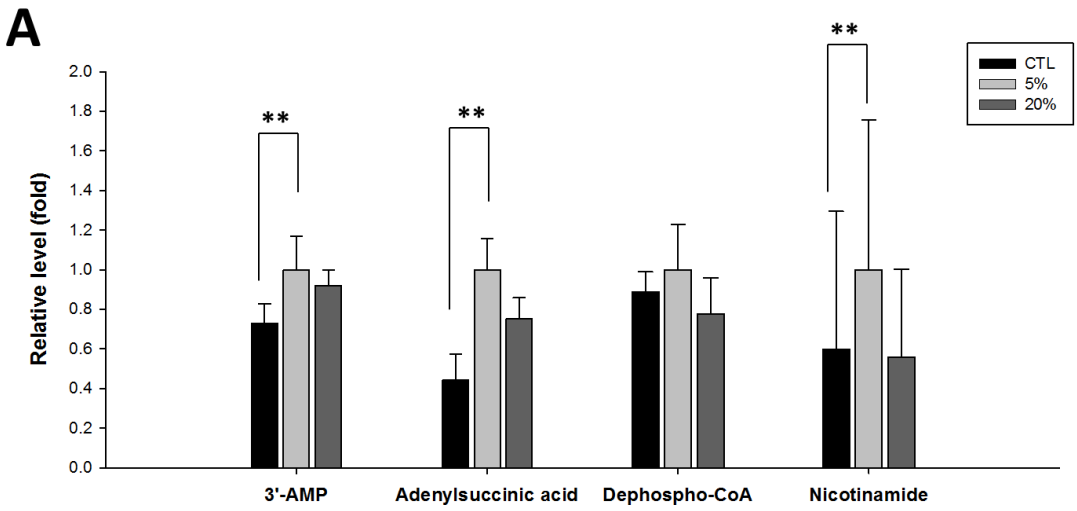


Figure 2.8 Figure 2.8 Effects of algae feeding on intermediate metabolites in nutrient and energy metabolism. The level of each metabolite marker is expressed as the fold of its highest level in three groups of mice. The highest level is artificially set as 1. ***A.*** Intermediate metabolites in the liver. ***B.*** Intermediate metabolites in urine. Values are means ± SD (n=8). Significant differences between groups are determined by the Bonferroni *t*-test and labeled as *($P < 0.05$) and **($P < 0.01$).



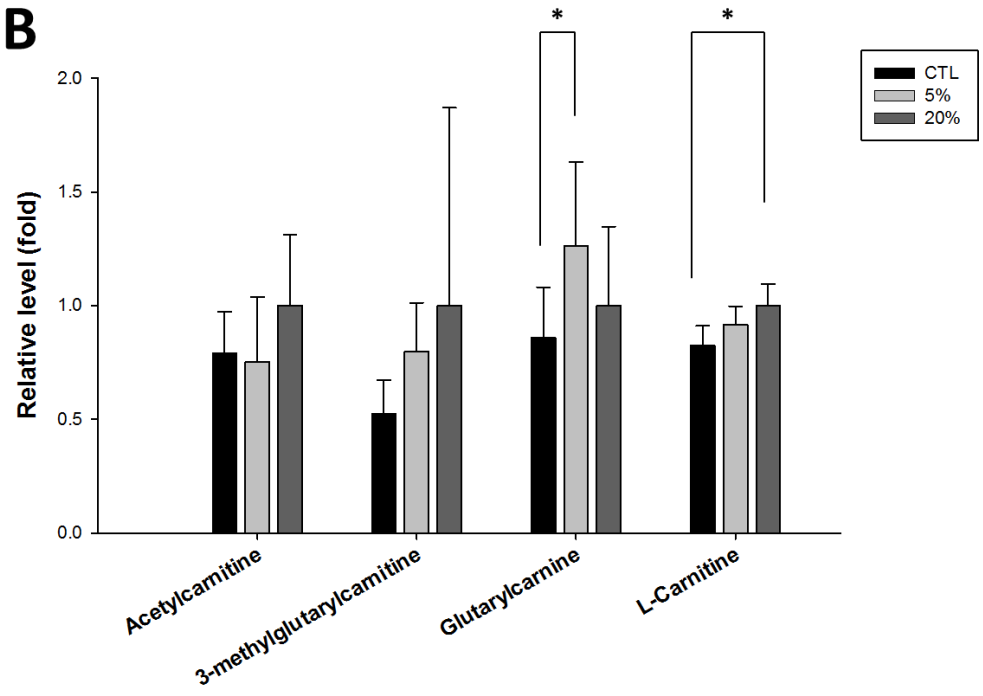
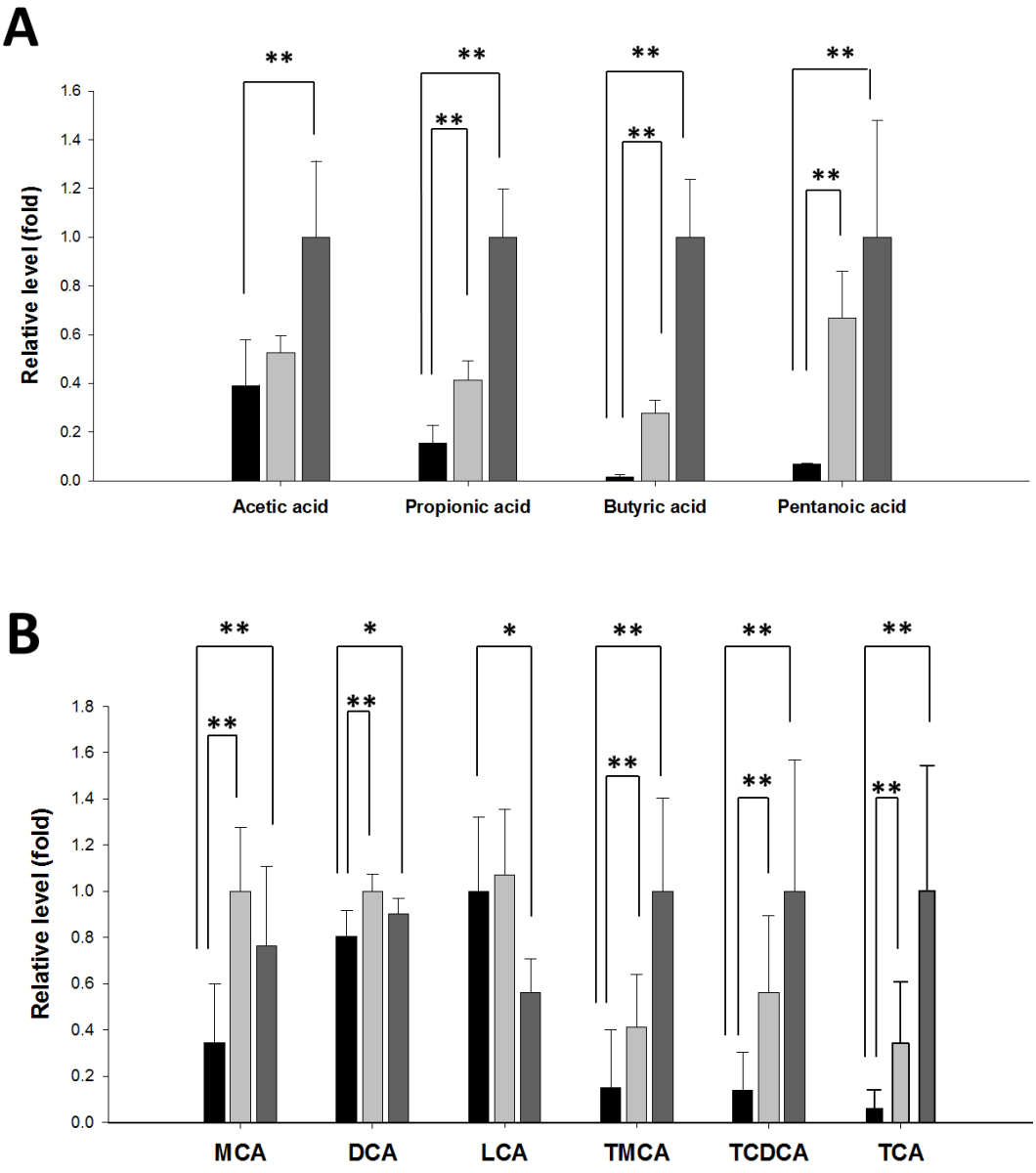
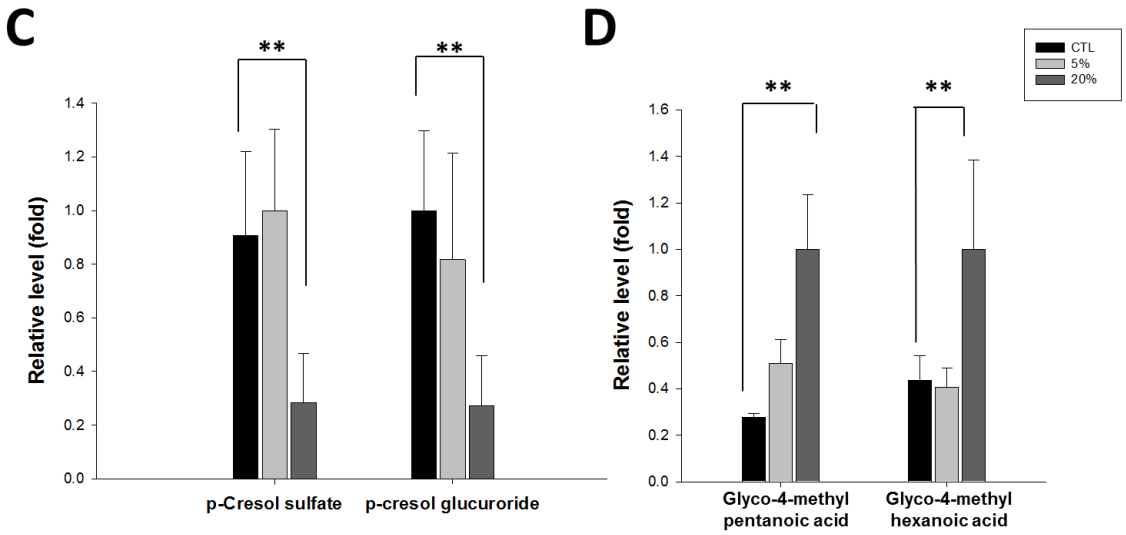

B
*
*
CTL
5%
20%
Relative level (fold)
2.0
1.5
1.0
0.5
0.0
Acetylcarnitine
3-methylglutarylcarnitine
Glutarylcarnine
L-Carnitine

Figure 2.9 Effects of algae feeding on microbial metabolites. The level of each metabolite marker is expressed as the fold of its highest level in three groups of mice. The highest level is artificially set as 1. ***A.*** SCFAs in feces. ***B.*** Bile acids and bile salts in feces. ***C.*** p-Cresol metabolites in urine. ***D.*** Glycine conjugates of branched fatty acids in urine. Values are means ± SD (n=8). Significant differences between groups are determined by the Bonferroni *t*-test and labeled as *($P < 0.05$) and **($P < 0.01$).




C
D
**
**
**
**
Relative level (fold)
Relative level (fold)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
p-Cresol sulfate
p-cresol glucuroride
Glyco-4-methyl pentanoic acid
Glyco-4-methyl hexanoic acid
CTL
5%
20%

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