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ALGAE PROVIDE OPTIONS FOR IMPROVED TREATMENT OF METABOLIC
SYNDROME

A Thesis submitted by

Ryan du Preez, BHealth, BScience (Hons)

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Abstract

Metabolic syndrome is a clustering of cardiometabolic risk factors including central obesity, hypertension, dyslipidaemia, glucose intolerance and non-alcoholic fatty liver disease which increases the risk of cardiovascular diseases and type 2 diabetes. Metabolic syndrome prevalence is increasing in all countries, for example in Australia, prevalence was 19% in 2000 and 29% in 2017 with an annual incidence of 3%. The continuing increase of incidence suggests that current treatment options are insufficient.

Seaweeds are part of the staple diet in south-east Asian countries and their intake correlates with a lower prevalence of metabolic syndrome. Seaweeds provide polysaccharides, vitamins, minerals, peptides, amino acids, polyphenols, carotenoids and omega-3 polyunsaturated fatty acids. This thesis investigated the physiological and metabolic responses to the whole biomass from species of red, green and brown macroalga (*Kappaphycus alvarezii* and *Sarconema filiforme*, *Caulerpa lentillifera*, and *Sargassum siliquosum* respectively) and microalgae (*Nannochloropsis oceanica*). Studies were performed in rats using a validated high-carbohydrate, high-fat diet with increased fructose and saturated/*trans* fats that induced cardiovascular, liver and metabolic signs, including obesity, hypertension, glucose/insulin intolerance and fatty liver, due to chronic low-grade inflammation. This diet induced changes that closely mimic the signs of human metabolic syndrome. The main components of interest in the seaweeds were the sulphated polysaccharides such as κ- and ι-carrageenans and non-sulphated polysaccharides such as fucoidans and alginates. As pigments often have low bioavailability, the advantage of nanoparticles was tested in curcumin.

Chapter 1 is a literature review of the therapeutic potential of macroalgae and microalgae as functional foods for metabolic syndrome. It also reviews curcumin and the application of nanoparticles to overcome low bioavailability. Chapter 2 determined that *K. alvarezii*, a red seaweed containing κ-carrageenan, normalised body weight and adiposity, lowered systolic blood pressure, improved heart and liver structure, and lowered plasma lipids in rats. In Chapter 3, *S. filiforme*, a red seaweed containing ι-carrageenan, decreased body weight by ~10%, abdominal fat deposits by ~30%, reduced systolic blood pressure by 13 mmHg, and reduced plasma liver enzyme activities. These red seaweeds have carrageenans which most likely act as prebiotics to attenuate symptoms of metabolic syndrome. Chapter 4 investigated *S. siliquosum*,

a brown seaweed, with alginate, fucoidans and laminarans as polysaccharides contributing to the 41.4% of dietary fibre in the biomass. It decreased body weight by ~12% but did not change other parameters. In Chapter 5, *C. lentillifera*, a green seaweed, decreased body weight by ~22%, reduced systolic blood pressure by 17 mmHg and reduced total plasma cholesterol and non-esterified fatty acids. The sulphated polysaccharides are the most likely compounds producing these physiological and metabolic actions. In Chapter 6, *N. oceanica*, a microalgae, was used as a source of eicosapentaenoic acid, up to ~9% dry weight for potential health benefits. A limiting factor was the intactness of the cell wall which may have decreased the bioavailability of eicosapentaenoic acid and hence optimal biological activity was not observed. In Chapter 7, a curcumin nanoparticle formulation was used to determine whether nanoparticles increase the low bioavailability of about 1% by 20-fold which may have future applications for other compounds with low bioavailability such as carotenoids from seaweeds.

Overall, this thesis found that seaweeds contain multiple compounds which attenuate obesity, hypertension, dyslipidaemia and inflammation, and therefore may be useful for further studies into functional foods for metabolic syndrome. A combination of seaweeds can be incorporated into the human diet to confer health benefits. Biotechnology applications such as nanoparticles can overcome low bioavailability which will lead to improved biological activity.

Keywords: diet-induced metabolic syndrome; functional foods; macroalgae; microalgae; polysaccharides; prebiotics; curcumin nanoparticles; gut microbiome

Certification of Thesis

This Thesis is the work of **Ryan du Preez** except where otherwise acknowledged, with the majority of the authorship of the papers presented as a Thesis by Publication undertaken by the Student. The work is original and has not previously been submitted for any other award, except where acknowledged.

Principal Supervisor: Dr Sunil K Panchal

Associate Supervisor: Professor Lindsay Brown

Student and supervisors' signatures of endorsement are held at the University.

Statement of Contribution

Chapter 2. κ-carrageenan in diet-induced metabolic syndrome.

Stephen Wanyonyi (SW), **Ryan du Preez (RDP)**, Lindsay Brown (LB), Nicholas A. Paul (NAP), Sunil K. Panchal (SKP).

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SW was responsible for 15% of conception and design, 20% collection of data, 30% of analysis and interpretation of data, 30% of drafting and writing and 10% of final important editorial input; RDP was responsible for 10% of conception and design, 50% collection of data, 30% of analysis and interpretation of data, 30% of drafting and writing and 10% of final important editorial input; LB was responsible for 30% of conception and design, 5% collection of data, 15% of analysis and interpretation of data, 15% of drafting and writing and 20% of final important editorial input; NAP was responsible for 10% of conception and design, 15% collection of data 10% of analysis and interpretation of data, 10% of drafting and writing and 15% of final important editorial input; SKP was responsible for 35% of conception and design, 10% collection of data, 15% of analysis and interpretation of data, 15% of drafting and writing and 45% of final important editorial input.

Chapter 3. ι-carrageenan in diet-induced metabolic syndrome.

Ryan du Preez, Nicholas A. Paul, Peter Mouatt (PM), Marwan Majzoub (MM), Torsten Thomas (TT), Lindsay Brown, Sunil K. Panchal.

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RDP was responsible for 40% of conception and design, 75% collection of data, 45% of analysis and interpretation of data, 50% of drafting and writing and 20% of final important editorial input; NAP was responsible for 20% of conception and design, 10% collection of data, 10% of analysis and interpretation of data, 10% of drafting and writing and 15% of final important editorial input; PM was responsible for 5%

collection of data; 5% of analysis and interpretation of data; MM was responsible for 5% of analysis and interpretation of data; TT was responsible for 5% of analysis and interpretation of data; LB was responsible for 20% of conception and design, 5% collection of data, 15% of analysis and interpretation of data, 20% of drafting and writing and 25% of final important editorial input; SKP was responsible for 20% of conception and design, 5% collection of data, 15% of analysis and interpretation of data, 20% of drafting and writing and 40% of final important editorial input.

Chapter 4. Brown seaweeds in diet-induced metabolic syndrome.

Ryan du Preez, Marie E. Magnusson (MEM), Rocky de Nys (RDN), Marwan Majzoub, Torsten Thomas, Christina Praeger (CP), Giuseppe C. Zuccarello (GCZ), Lindsay Brown, Sunil K. Panchal.

RDP was responsible for 30% of conception and design, 65% collection of data, 45% of analysis and interpretation of data, 50% of drafting and writing and 15% of final important editorial input; MEM was responsible for 10% of conception and design, 10% collection of data, 10% of analysis and interpretation of data, 5% of drafting and writing and 10% of final important editorial input; RDN was responsible for 10% of conception and design, 5% collection of data, 5% of analysis and interpretation of data, 5% of drafting and writing and 15% of final important editorial input; MM was responsible for 5% of analysis and interpretation of data; TT was responsible for 5% of analysis and interpretation of data; CP was responsible for 5% of conception and design, 5% collection of data, 5% of analysis and interpretation of data, 5% of drafting and writing; GCZ was responsible for 5% of conception and design, 5% collection of data, 5% of analysis and interpretation of data, 5% of drafting and writing a; LB was responsible for 20% of conception and design, 5% collection of data, 10% of analysis and interpretation of data, 15% of drafting and writing and 30% of final important editorial input; SKP was responsible for 20% of conception and design, 5% collection of data, 10% of analysis and interpretation of data, 15% of drafting and writing and 30% of final important editorial input.

Chapter 5. Green seaweeds in diet-induced metabolic syndrome.

Ryan du Preez, Lindsay Brown, Marwan Majzoub, Torsten Thomas, Sunil K. Panchal.

RDP was responsible for 60% of conception and design, 80% collection of data, 60% of analysis and interpretation of data, 55% of drafting and writing and 30% of final important editorial input; LB was responsible for 20% of conception and design, 10% collection of data, 20% of analysis and interpretation of data, 15% of drafting and writing and 30% of final important editorial input; MM was responsible for 5% of analysis and interpretation of data; TT was responsible for 5% of analysis and interpretation of data; SKP was responsible for 20% of conception and design, 10% collection of data, 10% of analysis and interpretation of data, 30% of drafting and writing and 40% of final important editorial input.

Chapter 6. Microalgae as a food intervention.

Ryan du Preez, Lindsay Brown, Marwan Majzoub, Torsten Thomas, Sunil K. Panchal.

RDP was responsible for 40% of conception and design, 75% collection of data, 55% of analysis and interpretation of data, 65% of drafting and writing and 30% of final important editorial input; LB was responsible for 30% of conception and design, 10% collection of data, 20% of analysis and interpretation of data, 15% of drafting and writing and 40% of final important editorial input; MM was responsible for 5% of analysis and interpretation of data; TT was responsible for 5% of analysis and interpretation of data; SKP was responsible for 30% of conception and design, 15% collection of data, 15% of analysis and interpretation of data, 20% of drafting and writing and 30% of final important editorial input.

Chapter 7. Curcumin nanoparticles in diet-induced metabolic syndrome.

Ryan du Preez, Jessica Pahl (JP), Meenakshi Arora (MA), M.N.V Ravi Kumar (RK), Lindsay Brown, Sunil K. Panchal.

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CHAPTER 1. INTRODUCTION: ALGAE AS FUNCTIONAL FOODS FOR METABOLIC SYNDROME

Algae are divided into macroalgae and microalgae. Macroalgae is a collective term for autotrophic, multicellular algae that require sufficient light to drive photosynthesis. The term includes seaweeds and other benthic marine algae that are generally visible to the naked eye but also includes freshwater species in inland seas (Chojnacka, Wieczorek et al. 2018). Larger macroalgae are referred to as seaweeds, although they are not really "weeds" (Diaz-Pulido and McCook 2008). Macroalgae are distinguished from microalgae such as diatoms, phytoplankton and the zooxanthellae that live in coral tissue, which require a microscope to be observed (Diaz-Pulido and McCook 2008). Microalgae are autotrophic, photosynthetic, unicellular algae that exist individually and can live in freshwater or marine environments. Macroalgae grow in littoral zones in every climate ranging for the tropical warm waters to the freezing cold polar regions (Holzinger and Karsten 2013) as well as in inland seas. More than 10,000 species of macroalgae have been discovered in the marine environment with only 1-5% used by animals and humans (Stabili, Acquaviva et al. 2012). Seaweeds are an important component of the diet in many Asian countries, especially Japan, Korea, China, Thailand and the Philippines but consumption is increasing in other countries. Global seaweed production was estimated at 30 million tonnes in 2016, growing at around 7.4% each year in the 2000s, with only 4.5% produced in the wild (listed] 2018). Most seaweed production is used for human consumption with the rest used for animal feeds, production of bioactive compounds and fertilisers. In 2014, China and Indonesia were by far the world's largest producers of seaweeds, each with more than 10 million tonnes. The next greatest producers were the Philippines and Korea, each with over 1 million tonnes. Japan, Malaysia and Zanzibar produced over 100,000 tonnes each (Buschmann, Camus et al. 2017) (Figures 1 and 2).

Macroalgae are divided into three main phyla according to their dominant pigmentation: Rhodophyta (red algae), Phaeophyta (brown algae) and Chlorophyta (green algae) (El Gamal 2010). Pigments include chlorophyll for green, phycobilins for red and fucoxanthin for brown algae (Dawczynski, Schafer et al. 2007). Macroalgae have a low-calorie value and contain carbohydrates, dietary fibre, proteins, vitamins, polyunsaturated fatty acids (PUFA), minerals and polyphenols

(Cardoso, Pereira et al. 2015, Wells, Potin et al. 2017). These may have therapeutic properties against metabolic diseases such as obesity and metabolic syndrome and have been proposed as a potential functional food (Mohamed, Hashim et al. 2012). Traditionally, seaweeds have been used in the food industry as gelling and thickening agents (Mohamed, Hashim et al. 2012). Cultivation of seaweed has the capacity to sustainably provide nutrient-rich food for human consumption (Tiwari and Troy 2015). In addition to a food source, seaweeds can be a considerable feedstock for biomass, biofuel production and animal feeds (Tiwari and Troy 2015).

Seaweeds are commonly found in Australian waters. Of the thousands of seaweed species found in southern Australian waters, over 60% do not occur anywhere else in the world (Agri Futures Australia 2017). Several species of seaweeds have marketable properties and are possibly suited to commercial cultivation in south-eastern Australia, with *Ulva* and *Porphyra* species showing the most promise (Agri Futures Australia 2017). Generally, the different types of seaweed have different chemical properties, and therefore different end-uses. Red seaweed is used for food and as sources of two hydrocolloids: agar and carrageenan. *Porphyra* species are the largest source of food from red seaweeds. Brown seaweed is mainly used as a source of the hydrocolloid, alginate, and the genera *Laminaria*, *Undaria* and *Hizikia* are used for food. These species do not exist in Australia and therefore alternative or equivalent species need to be chosen such as *Sargassum*. Green seaweed is used for foods or compounds such as *Caulerpa lentillifera* which has been commericalised for production by the James Cook University (Agri Futures Australia 2017).
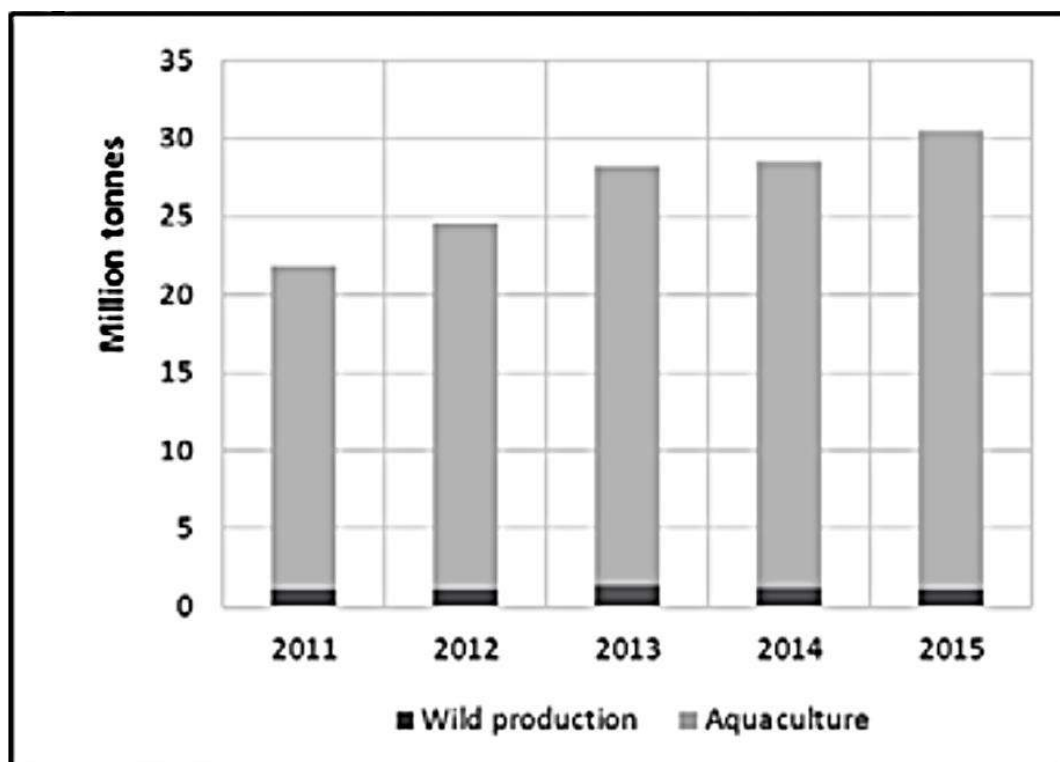
Figure 1. World production of seaweed during 2011-2015. Source: 2018 Food and Agriculture Organization of the United Nations (Fisheries and Aquaculture Department).
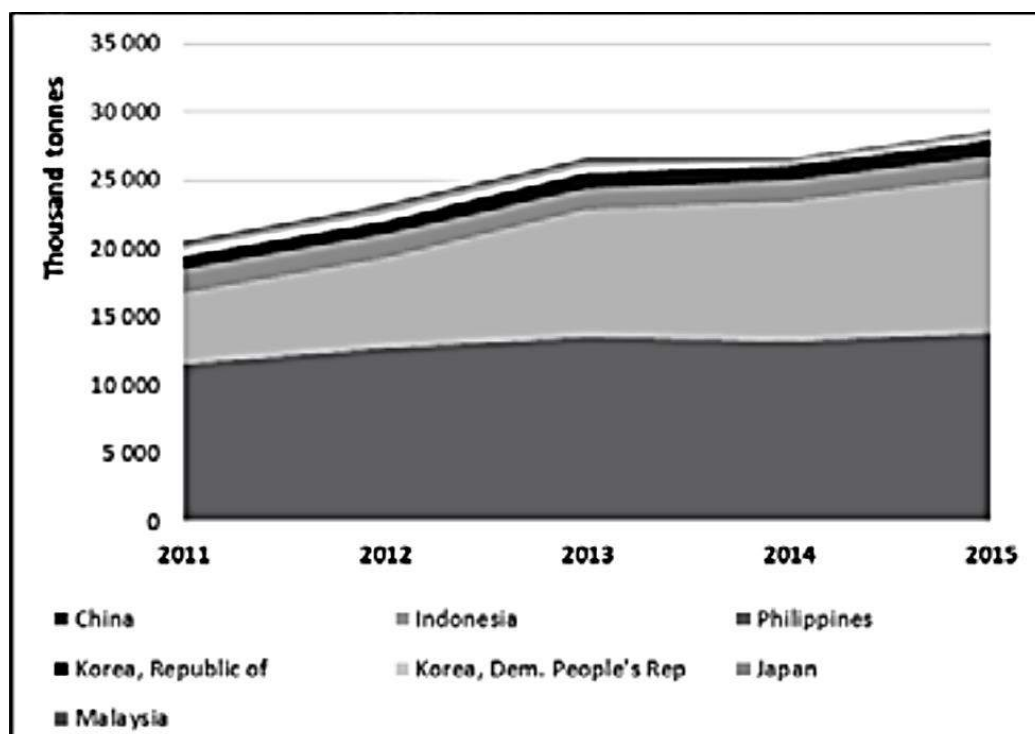

Figure 2. Asia: Leading producers of farmed seaweed during 2011-2015. Source: 2018 Food and Agriculture Organization of the United Nations (Fisheries and Aquaculture Department).

Health benefits from seaweeds

The highest consumption of seaweeds is traditionally in eastern and south-east Asian countries such as China, Korea, Japan, Vietnam and Indonesia. Seaweeds are considered an important part of a healthy diet and long life as evidenced by the average life expectancy in Japanese males of 77.10 and females of 83.99 years which is among the highest in the world (Yamori, Miura et al. 2001). Life expectancy is even greater in Okinawa, attributed to the low coronary heart disease and cancer mortalities, as a result of the Okinawan diet which is characterised by low salt, soy beans, fish, seaweed and green vegetables (Yamori, Miura et al. 2001). Seaweeds provide a complex natural resource of components including polysaccharides (dietary fibre), minerals, peptides, amino acids, polyphenols, carotenoids and $n-3$ PUFA. Consumption of seaweeds can match or enhance the supply of important nutrients from other readily available food sources for humans including oats and cereals for fibre, dairy products for calcium, fish for $n-3$ PUFA, and fruits and vegetables for carotenoids and polyphenols (MacArtain, Gill et al. 2007, Lordan, Ross et al. 2011, Khan, Anjum et al. 2013).

The potential therapeutic actions of these bioactive compounds in chronic diseases such as metabolic syndrome are recognised (Brown, Allsopp et al. 2014). Metabolic syndrome, described as a global epidemic (Saklayen 2018), is a grouping of symptoms including central obesity, insulin resistance, impaired glucose tolerance, dyslipidaemia and hypertension that increases the risk of type 2 diabetes mellitus, cardiovascular disease and many cancers (O'Neill and O'Driscoll 2015). Metabolic syndrome prevalence is high at 25-34% in the USA (Moore, Chaudhary et al. 2017) and lower in Japan at 8-12% (Kuzuya, Ando et al. 2007). China is a large producer of seaweeds with a metabolic syndrome prevalence of 8-10% (Lan, Mai et al. 2018). As an example of the potential of seaweeds, a healthy dietary pattern including green leafy vegetables and seaweeds in Korean adults decreased the development of metabolic syndrome by 24% over six years (Baik, Lee et al. 2013).

Compounds derived from seaweeds are effective functional foods and possible nutraceuticals (Ganesan, Tiwari et al. 2019). In particular, seaweeeds may be useful in controlling the signs of metabolic syndrome such as obesity, hypertension, type 2 diabetes, cardiovascular complications and hyperlipidaemia (Kumar and Brown 2013). Hence, there is strong evidence supporting seaweed as part of the human diet in more countries (Teas, Baldeon et al. 2009).

However, it is important to consider components of seaweeds, such as iodine and heavy metals, for potential harmful effects. For example, the Australian Quarantine and Inspection Service has included brown algae on the imported food "Risk List". They monitor imported products to ensure safe concentrations of iodine (<1000 mg/kg dried weight) (Food Standards Australia and New Zealand 2010). Excess iodine exposure from algae generally does not result in apparent clinical consequences, although vulnerable patients with specific risk factors may present with toxicity (Bouga and Combet 2015). Therefore, future studies should consider iodine concentrations in seaweed products.

Intakes of metals and metalloids such as aluminium, cadmium and lead through seaweed consumption do not raise serious health concerns (Khan, Ryu et al. 2015, Rubio, Napoleone et al. 2017). Seaweeds have been used as a low-cost adsorbent for the removal of pollutants from wastewater. The sulphated polysaccharides in the cellular wall of seaweeds allow for binding of pollutants such as trace metals (Arumugam, Chelliapan et al. 2018). There is great potential for low-cost micro and macroalgae cultivation for bioremediation (Zeraatkar, Ahmadzadeh et al. 2016). There is a well-documented review of acute methyl mercury poisoning following consumption of contaminated fish in Minamata, Japan from 1953 (Ekino, Susa et al. 2007). However, there are no reports of heavy metal toxicity in humans resulting from seaweed consumption. Furthermore, the consumption of 5 g/day of dehydrated seaweed would not pose a risk to the health of adults (Paz, Rubio et al. 2019).

Seaweeds appear to be a very minor source of vitamin K in Koreans, so potential additive risk with anticoagulant therapy seems remote. In a case report where a patient ate sushi wrapped in red seaweed, the authors concluded that intermittent changes in vitamin K should not necessitate permanent changes in the warfarin dosage (Bartle, Madorin et al. 2001). Additionally, seaweeds are rarely a major source of potassium in the diet and this is unlikely to lead to widespread health changes (McGrath, Harmon et al. 2010). Seaweeds may also be appropriate for biotechology applications by providing a high-yield, low-cost, non-toxic and eco-friendly option to produce nanoparticles for applications including biosensing, antimicrobial activity, anticancer activity, computer transistors, electrometers, chemical sensors, wireless electronic logic and memory schemes (Vijayan, Santhiyagu et al. 2016).

Macroalgae grown in different conditions

Macroalgae are found in tropical, temperate and freshwater with varying species and nutritional profiles. The freshwater alga, *Oedogonium intermedium* which grows in North Queensland, has carotenoids with activity against α-amylase, α-glucosidase and pancreatic lipase (Wang, Manabe et al. 2018). *Oedogonium* had the highest productivity (8.0 g ash free dry weight/m^2/day), lowest ash content (3-8%), lowest water content (fresh weight: dry weight ratio of 3.4), highest carbon content (45%) and highest bioenergy potential (higher heating value of 20 MJ/kg) compared to *Cladophora* and *Spirogyra* (Lawton, de Nys et al. 2013). Selecting the most suitable macroalgae for large-scale intensive cultivation is important to provide sufficient biomass for the wide range of applications including food, hydrocolloids, fertiliser, soil conditioner and biofuel (Lawton, de Nys et al. 2013).

Marine algae as dietary components are effective in the management of body weight and reduction of obesity (Wan-Loy and Siew-Moi 2016). The mechanism of action of anti-obesity actions is either through apoptosis in mature adipocytes or redistribution of adipose tissue. Further, brown adipose tissue thermogenesis may be an area of future research as a potential to stimulate energy expenditure for weight loss although, to date, studies have shown that brown adipose tissue contributes a small amount to overall energy metabolism (Marlatt and Ravussin 2017).

Seaweeds as sources of anti-obesity agents may work through prolonging gastric empying rate and thereby increasing satiety and thus reducing food consumption. Brown seaweeds have been the most successful at reducing body weight as demonstrated by alginates which decrease energy intake, thereby reducing body weight (Pelkman, Navia et al. 2007, Paxman, Richardson et al. 2008). Recent reviews extend these findings by emphasising that seaweeds have many nutritional benefits because they are low-fat, low-energy and have a high content of protein, fibre and polyphenols (Cherry, O'Hara et al. 2019, Cherry, Yadav et al. 2019). For example, on a gram-for-gram basis, seaweeds have protein and amino acid contents comparable to beef but seaweeds are currently consumed in smaller quantities so their effects are not maximised (Cherry, O'Hara et al. 2019). Currently, research has focussed on studies in rodents and hence human clinical trials are needed to show long term efficacy of seaweed products.

Commercial uses of algae

Seaweed-derived polysaccharides such as alginates, carrageenans and agars have been used for decades for their emulsifying, stabilising and thickening characteristics to improve the sensory properties of food. These uses could continue to occur which would help offset the costs for using seaweeds for health, diverting some produce from commercial uses to those for health benefits. More recently, there has been interest in their application as functional dietary fibre (Cherry, O'Hara et al. 2019). Seaweed-derived peptides, $n-3$ fatty acids, carotenoids, polyphenols and pigments have also attracted interest due to their wide anti-inflammatory therapeutic activity which is beneficial against metabolic syndrome. Hence, seaweeds may be used as a functional food. The International Life Sciences Institute defines functional foods as "foods that, by virtue of the presence of physiologically-active components, provide a health benefit beyond basic nutrition" (listed] 1999). Suitable treatment options from natural products that can provide effective treatment against the increasing health problems of obesity is a priority (Kumar, Magnusson et al. 2015). The potentially bioactive compounds from seaweeds including polysaccharides, peptides, pigments, minerals and $n-3$ fatty acids may have therapeutic potential against metabolic syndrome (Kumar and Brown 2013). The next section will explore the animal and human studies of red, brown and green seaweeds.

Red seaweeds

Red seaweeds contain the polysaccharides, agar and carrageenans, and the pigments, phycoerythrin and phycocyanin (Yende, Harle et al. 2014). Agar is a polysaccharide composed of agarose and agaropectin. Chemical or enzymatic hydrolysis of agar yield red algae oligosaccharides (Cheong, Qiu et al. 2018). The health benefits include prebiotic, immuno-modulatory, anti-inflammatory, anti-oxidant and anti-tumour effects (Cheong, Qiu et al. 2018). A prebiotic is a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health (Gibson, Scott et al. 2010). Gut dysbiosis is the change in the microbial composition that causes a drastic imbalance between the beneficial and potentially pathogenic bacteria. Therefore, the gut becomes vulnerable to pathogenic insult with gut microbial alterations (DeGruttola, Low et al. 2016). For example, high-fat diet-induced gut dysbiosis was prevented by administering a mixture of prebiotic agaro-

oligosaccharides, which reportedly averts a change in the microbial composition induced by consumption of a high-fat diet in C57BL/6N mice (Higashimura, Naito et al. 2016). The ι-, κ- or λ-carrageenans are linear polysaccharide with one, two or three sulphates per disaccharide unit, respectively. Carrageenans have a wide range of therapeutic uses such as anti-coagulants and anti-thrombotics (Pomin 2010). There is increasing interest in using these compounds for their health benefits. The physiological effects of red seaweed oligosaccharides may be attributed to their chemical and physical properties, such as monosaccharide composition, linkage type, glycosidic bond linkages, sulphation and molecular weight (Cheong, Qiu et al. 2018). Anti-obesity effects of *Kappaphycus alvarezii* have been reported in mice (Chin, Mi et al. 2019). In this study with diet-induced obese C57BL/6J mice, extracted native κ-carrageenan and the leftover fraction sans-carrageenan at 5% doses were more effective at reversing obesity and related metabolic syndromes than the whole *K. alvarezii*. The possible mechanism was reported to be carrageenan, as the major soluble fibre, having multiple actions including increased lipolysis and decreased lipogenesis resulting in reduced body weight and fat. Another study found an extract from *Polysiphonia japonica* may be a potential functional food because it protected insulin-secreting *β*-cells from toxicity induced by palmitate (Cha, Kim et al. 2018).

Brown seaweeds

Brown seaweeds including *Sargassum* species are a rich source of bioactives such as fucoidan, phlorotannins, and xanthophyll and fucoxanthin pigments (Li, Wijesekara et al. 2011). The main pigment in brown seaweeds is the carotenoid fucoxanthin and the main polysaccharides are fucoidan, alginate and laminaran (Liu, Heinrich et al. 2012). Bioactives such as meroterpenoids, phlorotanins, fucoidans, sterols and glycolipids have been identified in *Sargassum* species (Liu, Heinrich et al. 2012). There are numerous therapeutic and health benefits from *Sargassum* species including anti-inflammatory, anti-oxidant, anti-coagulant and hepatoprotective effects (Yende, Harle et al. 2014). An extract of *Ishige okamurae*, a brown seaweed, had *in vitro* activity by inhibiting lipid accumulation, which is induced during adipogenesis from 3T3-L1 preadipocytes (Yang, Fernando et al. 2019). Further to this anti-obesity activity, the same extract had anti-diabetic activity demonstrated by inhibiting *α*-amylase and *α*-glucosidases which regulate glucose absorption. This activity was attributed to the action of phlorotannins.

A randomised, double-blind, placebo-controlled, four-way crossover intervention study with 15 g of sodium alginate, containing 9.5 g of fibre, in 500 mL of water for four days separated by washout period of >3 weeks reported the participants had increased satiety feelings, reduced hunger and urge of future food consumption. Additionally, energy intake was reduced by 5.5% (Jensen, Kristensen et al. 2012). In another study, 5 g/day of wakame (*Undaria pinnatifida*) for eight weeks in hypertensive subjects reduced systolic and diastolic blood pressure (Hata, Nakajima et al. 2001). The anti-hypertensive effect was attributed to the alginate content.

Green seaweeds

Green seaweeds including *Caulerpa* species have sulphated polysaccharides different to land plants (Alves, Caridade et al. 2010) which exhibit therapeutic properties including reversing metabolic syndrome in rats (Kumar, Magnusson et al. 2015). Sea grapes include high anti-oxidant concentrations (Nguyen, Ueng et al. 2011). Green seaweeds are dominated by chlorophyll a and b, with ulvan being the major polysaccharide component (Yende, Harle et al. 2014). Two tropical green seaweeds, *Ulva ohnoi* and *Derbesia tenuissima*, reversed metabolic syndrome induced by a high-carbohydrate, high-fat in Wistar rats (Kumar, Magnusson et al. 2015). *U. ohnoi* contained 40.9% total fibre, of which 18.1% was soluble fibre, and higher magnesium content. *U. ohnoi* decreased body fat mass by 24%, systolic blood pressure by 29 mmHg and improved glucose utilisation and insulin sensitivity. *D. tenuissima* contained 23.4% total fibre, essentially as insoluble fibre, and decreased plasma triglycerides by 38% and total cholesterol by 17%. *U. ohnoi* was more effective than *D. tenuissima* in reducing metabolic syndrome, possibly due to the increased intake of soluble fibre and magnesium. Chlorophylls and their derivatives have been used in medicine for anti-oxidant properties and the food industry as colourants (Solymosi and Mysliwa-Kurdziel 2017). For example, subjects who consumed 5 g of chlorophyll daily for 12 weeks lost weight, had reduced obesity-related risk-factors and had a decreased urge for palatable food (Montelius, Erlandsson et al. 2014).

Seaweed-derived polysaccharides

Sulphated polysaccharides are part of the seaweed cell wall that provide support and defence to the seaweed including carrageenans, fucoidans and ulvans (Figure 3) which have therapeutic potential (Rhein-Knudsen, Ale et al. 2015, Gómez-Guzmán, Rodríguez-Nogales et al. 2018).
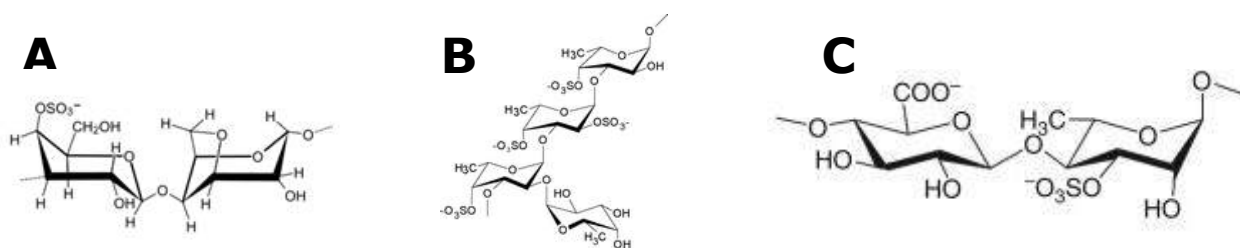
Figure 3. Chemical structures of the sulphated polysaccharides, κ-carrageenan (A), fucoidan (B) and ulvan (C).

Carrageenans

Carrageenans are mainly isolated from red algae and used in the food industry for their thickening, gelling and stabilising properties (Cunha and Grenha 2016). κ-carrageenan is the major type used in the food industry. It cannot be fermented by the human microbiota when it has a molecular weight of between 100 kDa to 450 kDa (Vo, Lynch et al. 2019). However, a low molecular weight polysaccharide of 4.5 kDa was metabolised by the human gut microbiota (Vo, Lynch et al. 2019). Carrageenan cannot be hydrolysed by gastric and intestinal juices and is also inert to hydrolysis by human intestinal enzymes (Michel and Macfarlane 1996). Hence, metabolism of orally administrated carrageenan is largely performed by gut microbiota. It is generally accepted that a low molecular weight carrageenan called poligeenan is a pro-inflammatory agent that is toxic to the colon (Tobacman 2001). However, degraded carrageenans which are not produced biologically have been incorrectly referred to as poligeenans. Poligeenans are produced in the laboratory or commercially by subjecting carrageenan to very low pH (0.9-1.3) and non-physiological temperatures (>80°C) for several hours (McKim, Willoughby et al. 2018. doi: 10.1080/10408398.2018.1481822). As carrageenans are not absorbed from the gastrointestinal tract after oral administration, studies using systemic administration are not appropriate for an assessment of risk of carrageenans when used as food products (Cohen and Ito 2002).

Alginates

Alginates are linear water-soluble polysaccharides containing 1,4-linked chains of *α*-L-guluronic acid and *β*-D-mannuronic acid as the major sugar residues. There are variations in the mannuronic/guluronic acid ratios and linear arrangements.

They are the most abundant polysaccharide in brown algae, with high concentrations in some seaweeds, for example, the alginate content of a number of seaweeds, based on dry weight, is as follows: *Ascophylum nodosum*, 22-30%; *Laminaria digitata* fronds, 25-44%; *L. digitata* stipes, 35-47%; *L. hyperborea* fronds, 17-33% and *L. hyperborea* stipes, 25-38%. Alginate contents of between 17-45% have been reported in *Sargassum* species (O'Sullivan, Murphy et al. 2010). As a dietary fibre, alginates are readily fermented by *Bacteroides ovatus*, a specific gut bacterium. This fermentation produces short chain fatty acids (SCFA), a critical source of energy for intestinal epithelial cells and immune cells (Chambers, Preston et al. 2018). Alginates have inhibitory activity against pancreatic lipase with the inhibitory activity varying with the source and chemical forms of the alginate. Alginates from *Laminaria hyperborea,* with high glucoronic acid content, showed higher inhibition of pancreatic lipase than alginates from *Lessonia nigrescens,* with high mannuronic acid content (Wilcox, Brownlee et al. 2014).

Fucoidans

Fucoidans are a family of sulphated fucose-rich polysaccharides, containing a backbone of α-linked L-fucose units and one or more residues of xylose, mannose, galactose, rhamnose, arabinose, glucose or glucuronic acid and acetyl groups in a variety of brown algae (Ale and Meyer 2013). Algae such as *Fucus vesiculosus*, *Sargassum stenophyllum*, *Chorda filum*, *Ascophyllum nodosum*, *Dictyota menstrualis* and *Caulerpa racemosa* contain fucoidans (Luthuli, Wu et al. 2019). As a highly-sulphated macromolecule, fucoidan cannot be digested by human or bacterial enzymes (Nagamine, Nakazato et al. 2014). Hence, dietary fucoidan has prebiotic-like effects, which increased the abundance of *Lactobacillus* species and reduced *Enterobacterium* species in the proximal and distal colon of pigs (Lynch, Sweeney et al. 2010). Fucoidan extracted from *F. vesiculosus* has been used as a α-glucosidase inhibitor that is able to treat diabetes (Shan, Liu et al. 2016). Fucoidan also improves glucose tolerance by modulating AMP-activated protein kinase signalling and glucose transporter 4 activity (Jeong, Kim et al. 2013). Fucoidan extracted from the sea cucumber, *Acaudina molpadioides*, was tested in high-fat, high-sucrose diet-induced hyperglycaemia and insulin resistant mice. The extract from *A. molpadioides* at a dose of 80 mg/kg/day for 19 weeks improved oral glucose tolerance, reduced body weight,

reduced hyperlipidaemia and protected the liver from steatosis (Hu, Xia et al. 2014). Taken together, these actions are useful for treating metabolic syndrome.

Ulvans

Ulvans are water-soluble sulphated polysaccharides mainly extracted from *Ulva* species, a green seaweed. Ulvans account for 18-29% of the algal dry weight (Alves, Sousa et al. 2013). These polysaccharides are mainly composed of glucuronic acid and iduronic acid units together with rhamnose and xylose sulphates, connected by α- and β-1→4 bonds, with the molecular weight of ulvans ranging from 150 kDa to 2000 kDa (Pérez, Falqué et al. 2016). Currently, these compounds are used in the food industry for their gelling and thickening capabilities (Bixler and Porse 2011). However, they may also be useful as high-fibre sources (O'Sullivan, Murphy et al. 2010) for the attenuation of metabolic syndrome symptoms. Unique features of the marine environment differentiate the properties of seaweeds from terrestrial plants. Seaweeds are rich in bioactive compounds which have health-promoting benefits (Rajapakse and Kim 2011). Hence, seaweeds may have an role in modulating human disease states (Brown, Allsopp et al. 2014). Other applications of seaweeds include increasing shelf-life and as a good source of macro- and micronutrients such as iodine (Roohinejad, Koubaa et al. 2017). Nanoparticles from nautral polymers have been used for pulmonary drug delivery (Carter and Puig-Sellart 2016). Polysaccharides are considered safe, biocompatible, stable, hydrophilic and biodegradable, and they can be modified into different forms, such as chemically modified polysaccharides, hydrogels, scaffolds, fibres and nanoparticles (Venkatesan, Anil et al. 2016).

Seaweed-derived peptides

Protein concentrations in seaweeds range from 5% to 47% of dry matter and depend particularly on species and the environmental conditions (Cerna 2011). Seaweed protein is a source of all amino acids, especially glycine, alanine, arginine, proline, glutamic and aspartic acids (Cerna 2011). In algae, essential amino acids represent about half the total amino acids and their protein profile is similar to eggs, a well-known source of protein (Kuang, Yang et al. 2018). Algal protein extraction methods include enzymatic hydrolysis, physical processes and chemical extraction (Bleakley and Hayes 2017). Other newer methods such as ultrasound-assisted extraction, pulsed electric field and microwave-assisted extraction have also been used (Bleakley and Hayes 2017). For nutritional purposes, amino acid analysis of

macroalgae shows brown macroalgae is generally low, usually below 150 g kg^{-1} of dry matter, whereas green macroalgae and especially red macroalgae, have a higher protein content. Some red macroalgae, such as *Porphyra* species have protein levels comparable to soybean meal (Øverland, Mydland et al. 2019). Peptides derived from natural sources such as seaweeds are worth investigating as they do not have negative side effects of drugs such as synthetic angiotensin converting enzyme (ACE) inhibitors (Bougatef, Nedjar-Arroume et al. 2008). The macroalgae, *Ulva intestinalis*, was hydrolysed by different proteases and the trypsin hydrolysate exhibited the highest level of ACE inhibitory activity (Sun, Xu et al. 2019). Bioactive peptides from *Porphyra* species, a type of red reaweed, were identified from proteolytic enzymes hydrolysates (Admassu, Gasmalla et al. 2018). The resultant fractions inhibited *α*-amylase activity by 88.67 ± 1.05% in *in vitro* porcine pancreas. This reduced glucose concentration indicates these novel peptides have potential for development of health promoting ingredients in food and pharmaceuticals for diabetes management.

Seaweed-derived omega-3 fatty acids

PUFA from microalgae are valuable commercial products with an estimated market value of 140 USD/kg in 2013 (Borowitzka 2013). The PUFA group includes *n*−3 fatty acids which have demonstrated action against inflammation, cardiovascular diseases and mental disorders (van Ginneken, Helsper et al. 2011); which may be reversed by the presence of *n*−6 fatty acids exerting neutral or negative effects (Poudyal and Brown 2015). An increased consumption of fat and vegetable oils rich in *n*−6 fatty acids coupled with a decreased consumption of foods rich in *n*−3 fatty acids has resulted in a *n*−6:*n*−3 ratio of up to 20:1 in Western diets compared to a ratio of about 1:1 in the diet of our ancestors (Molendi-Coste, Legry et al. 2011). A high ratio of *n*−6:*n*−3 fatty acids restricts the associated health benefits of *n*−3 fatty acids because both have the same rate-limiting enzymes. The *n*−3 PUFA have a double bond between the third and fourth carbon atoms from the methyl end of the fatty acid. The *n*−3 PUFA can be either short chain with ≤ C18 or long chain with ≥ C20 (Ryckebosch, Bruneel et al. 2014). The health benefits of *n*−3 long chain-PUFA are mainly due to eicosapentaenoic acid (EPA, 20:5 *n*−3) and docosahexaenoic acid (DHA, 22:6 *n*−3) (Gogus and Smith 2010). The lipid content of algal cells naturally varies between 1-70% but can reach 90% of dry weight under certain conditions (Metting 1996). Humans are unable to endogenously synthesise PUFA exceeding 18 carbon atoms.

However, because of their known benefits to human health, PUFA are part of a recommended diet such as fatty fish (Molendi-Coste, Legry et al. 2011). Fishes such as salmon, mullet and mackerel are the main sources of EPA and DHA for human consumption (Whitehead 1985, Gunstone 1996) and accumulate PUFA by feeding on microalgae that are rich in lipids and fatty acids.

Seaweed-derived carotenoids

Carotenoids are naturally occurring fat-soluble pigments, that provide colours such as yellow, red and orange, in fruits and vegetables, plants, algae and photosynthetic bacteria (Khoo, Prasad et al. 2011). Humans cannot synthesis carotenoids and therefore depend on dietary intake. The role of carotenoids in human health are primarily as anti-oxidants. Carotenoids are most bioavailable when they are consumed with foods with a lipid base such as egg yolks and when blended or heated which breaks down cell walls of the plant (Fernández-García, Carvajal-Lérida et al. 2012). These lipophilic molecules are traditionally grouped based on their chemical structure into carotenes and xanthophylls. Both groups have a common C40 polyisoprenoid structure containing a series of centrally located, conjugated double bonds which act as a light-absorbing chromophore (Khoo, Prasad et al. 2011). Carotenoids that exist as pure non-polar hydrocarbons are referred to as carotenes (α-carotene, β-carotene and lycopene); on the contrary, xanthophylls (β-cryptoxanthin, lutein, zeaxanthin and astaxanthin) are more polar carotenoids that contain oxygen as a functional group in its structure either as a hydroxyl or keto group as the end group. The polar group in the structure affects the polarity and biological function of the compound (Deming and Erdman 1999). There are numerous health benefits of carotenoids related to the pathophysiology of non-alcoholic fatty liver disease (NAFLD) (Lee, Hu et al. 2019). NAFLD occurs when lipid homeostasis is dysregulated resulting in excessive fat accumulation in the liver. Consequently, in this abnormal physiological state, the liver becomes suscpetibilty to oxidative stress, inflammation, lipotoxicity and apoptosis which increases liver damage.

Astaxanthin (Figure 4A) is another high-value product derived by microalgae. This carotenoid can be produced naturally or synthetically. In 2014, the commercial market of synthetic astaxanthin was valued at 447 million USD, corresponding to the production of 280 tonnes (~1600 USD/kg) (Koller, Muhr et al. 2014, Panis and Carreon 2016). *Haematococcus pluvialis* is a natural source of astaxanthin. This

microalgae can be used as a biorefinery for increased production through conventional biorefinery methods such as mechanical disruption, solvent extraction, direct extraction using vegetable oils and enhanced solvent extraction. However, recent advancements in biorefinery extractions including supercritical CO_2, magnetic-assisted, ionic liquids and supramolecular solvents may improve production volumes and value (Khoo, Lee et al. 2019). Astaxanthin has great demand in food, feed, nutraceutical and pharmaceutical applications. This has promoted major efforts to improve astaxanthin production from biological sources instead of synthetic procedures (Ambati, Phang et al. 2014). Biological sources are preferred because natural astaxanthin is more than 95% esterified whereas synthetic astaxanthin is un-esterified. Astaxanthin products are available in the form of capsule, soft gel, tablet, powder, biomass, cream, energy drink, oil and extract (Ambati, Phang et al. 2014). Peroxisome proliferator-activated receptor (PPAR) is involved in lipid and carbohydrate metabolism as well as the management of metabolic syndrome and related disorders such as obesity, type 2 diabetes, atherosclerosis and NAFLD. Astaxanthin is a PPARα agonist and PPARγ antagonist and has a physiological role in hepatic lipid and glucose metabolism (Choi 2019). Astaxanthin blocked the rapid-onset oxidative stress, inflammatory pathway and neuronal death during status epilepticus, demonstrating neuroprotective effects of astaxanthin (Fakhri, Aneva et al. 2019). Based on these actions, it has been suggested that marine carotenoids could provide physiological benefits in human health (Chuyen and Eun 2017). For example, a placebo-controlled human trial using doses of 0, 6, 12 or 18 mg/day of astaxanthin for 12 weeks in non-obese subjects with mild hyperlipidaemia and without diabetes or hypertension was conducted (Yoshida, Yanai et al. 2010). It found that 12 and 18 mg/day reduced serum triglycerides and increased adiponectin while 6 and 12 mg/day increased HDL-cholesterol (Yoshida, Yanai et al. 2010). Daily administration of 6 mg/kg body weight of astaxanthin for 60 days attenuated diet-induced obesity, hepatic steatosis and hepatic TGF-β1 protein levels in mice (Bhuvaneswari, Arunkumar et al. 2010). Improving the lipid profile and the regulation of glucose and fatty acid metabolism are important parameters in attenutating metabolic syndrome.

Another xanthophyll, fucoxanthin (Figure 4B) from brown seaweed, demonstrated anti-obesity activity in mice. Fucoxanthin has anti-obesity effects, improves the plasma lipid profile and liver function in rodents fed a high-fat diet (Muradian, Vaiserman et al. 2015). The primary mechanism has been reported as

fucoxanthin-induced uncoupling protein-1 in abdominal white adipose tissue, leading to the oxidation of fatty acids and heat production, thus decreasing body weight (Gammone and D'Orazio 2015). Further, fucoxanthin improved insulin resistance and ameliorated blood glucose concentrations through down-regulation of adipocytokines related to insulin resistance in white adipose tissue and up-regulation of glucose transporter 4 in skeletal muscle (Maeda 2015).
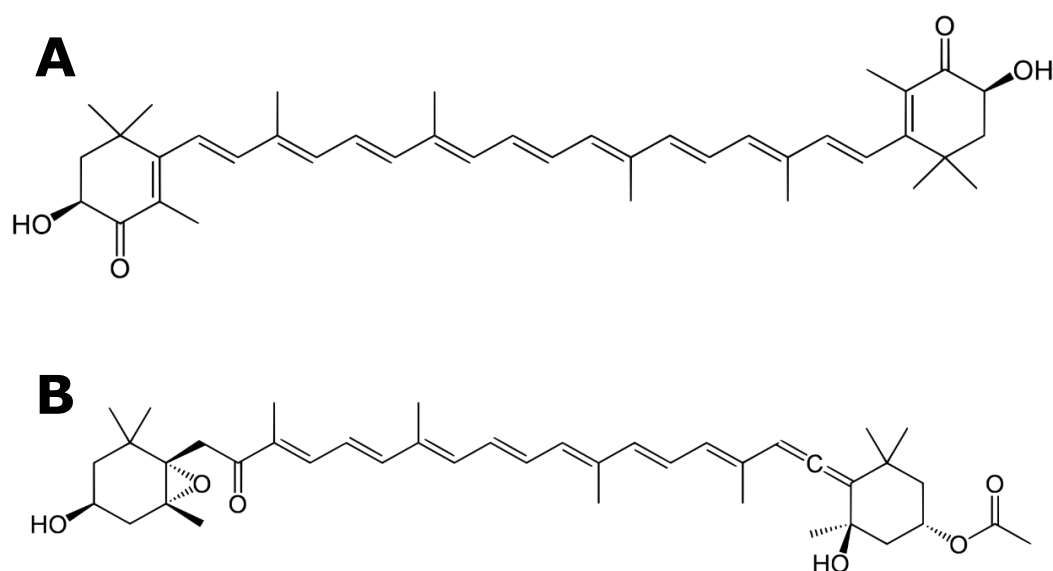
Figure 4. Chemical structures of astaxanthin (A) and fucoxanthin (B).

Seaweed-derived polyphenols

Polyphenols are secondary metabolites of plants and seaweeds grouped according to their structure, into phenolic acids, flavonoids, stilbenes, lignans and others phenolic compounds. Polyphenols are the major group of phytochemicals found in the human diet, sourced from fruits, vegetables, seeds, essential oils and their derived foods and beverages (Gómez-Guzmán, Rodríguez-Nogales et al. 2018). The potential anti-oxidant activity from polyphenols derived from seaweeds has been reviewed (Jacobsen, Sørensen et al. 2019). Marine macroalgae containing polyphenols with potential health benefits are associated with longevity and seaweeds are widely regarded as having the potential to prevent or exert beneficial effects on different diseases, including cardiovascular-associated disorders such as hypertension, type 2 diabetes and obesity. Although polyphenols have very low bioavailability due to their reduced absorption and rapid metabolism, there have been several epidemiological studies which found an inverse association between polyphenols and different diseases (Gómez-Guzmán, Rodríguez-Nogales et al. 2018).

Phlorotannins are polyphenolic compounds with a wide range of molecular weights (126 kDa to 650 kDa), which have been reported for almost all brown seaweed orders (Lopes, Andrade et al. 2016). Brown seaweed extracts of phlorotannins have been explored for their ability to suppress carbohydrate digestion and glucose absorption and hence have anti-diabetic action. The structure of phlorotannins are similar to α-glucosidase and hence both have similar actions in carbohydrate metabolism.

Seaweed-derived pigments

Algae contain photosynthetic pigments that are usually an integral part of the structure of the chloroplast lamellae with function to absorb light to carry out photosynthesis. Algal pigments are categorised into three classes: chlorophylls, carotenoids and phycobiliproteins (Galasso, Gentile et al. 2019). Chlorophylls are the major photosynthetic pigments in algae, plants and cyanobacteria. Chlorophyll a-d are four subtypes of the chlorophyll pigments that absorb different wavelengths. Chlorophyll a is the most important pigment, with a maximum absorbance from 660-665 nm, essential for photosynthesis because it transports energised electrons to produce sugars. Chlorophyll b is an accessory pigment which acts indirectly during photosynthesis by absorbing and transferring energy to chlorophyll a. Chlorophyll b is found in green algae with a maximum absorbance from 642-652 nm. Chlorophylls c and d are accessory pigments with maximum absorbances from 447-452 nm and 710 nm, respectively. Chlorophyll d is found in red algae (Manivasagan, Bharathiraja et al. 2018). Marine natural pigments have a wide therapeutic potential including anti-oxidant, anti-obesity, anti-inflammatory and anti-cancer activities (Manivasagan, Bharathiraja et al. 2018).

Microalgae

Microalgae are microscopic, single cellular photosynthetic organisms that grow in a range of aquatic habitats, including lakes, pounds, rivers, oceans and wastewater (Khan, Shin et al. 2018). Examples of microalgae genera are *Arthrospira* (formerly known as *Spirulina), Chlorella, Nannochloropsis* and *Scenedesmus* (Duran, Kumar et al. 2018. doi: 10.1080/17597269.2018.1457314). They are a rich source of carbon compounds, which can be used in biofuels, health supplements, pharmaceuticals and cosmetics (Das, Aziz et al. 2011). Microalgae are a source of high-value compounds such as carotenoids, PUFA, protein, anti-oxidants and

pigments (Yaakob, Ali et al. 2014). Microalgae also represent a valuable source of nearly all essential vitamins, including pro-vitamin A (*α*- and *β*-carotene and apocarotenoids), vitamin C (ascorbic acid), vitamin E (tocopherols and tocotrienols), vitamin D and some vitamins of the B group such as B1 (thiamine), B2 (riboflavin), B3 (niacin) and B12 (cobalamin) (Becker 2004). Vitamins exhibit strong anti-oxidant activity and are essential for health as precursors of enzyme cofactors for essential metabolic functions. Since most vitamins cannot be endogenously synthesised in humans, they need to be taken orally (Galasso, Gentile et al. 2019). The balanced nutritional and bioactive profile of microalgae species has demonstrated therapeutic effects against chronic diseases such as metabolic syndrome (Koyande, Chew et al. 2019). Microalgae have been used in products such as biscuits, breads, yoghurt and pasta with demonstrated anti-oxidative and anti-hypertensive effects (Koyande, Chew et al. 2019). Hence, microalgae may be used as functional foods against several diseases states implicated in metabolic syndrome.

Microalgae can metabolise or bio-transform chemicals, for example, their potential against surfactants, wastewater and herbicides has been demonstrated. The potential of using the microalgae as a tool and profit from it is significant and yet to be realised (Sosa-Hernandez, Romero-Castillo et al. 2019). Micoalgae may form part of the solution to a global increased demand of protein in the form of sustainable aquafeeds to provide alternative sources of protein (Yarnold, Karan et al. 2019). *Chlorella* contains anti-oxidants such as lutein, *α*-carotene, *β*-carotene, ascorbic acid and *α*-tocopherol, which are active against free radicals, a common hallmark of many diseases (Bhattacharjee 2016). *Chlorella vulgaris, C. ellipsiodea, Arthrospira platensis* and *Nannochloropsis oculata* have been used to produce ACE-inhibitory peptides (Ejike, Collins et al. 2017). Dry *Chlorella* contains 55-67% protein, 1-4% chlorophyll, 9-18% dietary fibre as well as minerals and vitamins. It also contains lutein which has reported anti-inflammatory properites. Lutein can improve or prevent age-related macular disease which is the leading cause of blindness and vision impairment (Buscemi, Corleo et al. 2018). These properties and therapeutic benefits support the use of *Chlorella* as a promising potential functional food (Matos, Cardoso et al. 2017). *Arthrospira* is a valuable source of fatty acids such as palmitic, oleic, lauric and DHA which accounts for up to 9.1% of the total fatty acid content. *Arthrospira* has positive health effects in weight loss, diabetes and hypertension (Matos, Cardoso et al. 2017). *Haematococcus pluvialis* is the largest natural source of

astaxanthin accounting for up to 3% of its dry weight (Jaime, Rodríguez-Meizoso et al. 2010). This pigment has anti-oxidant, anticancer, anti-inflammatory and antibacterial properties (Lordan, Ross et al. 2011). Microalgae derivatives have shown potential as cancer prevention agents (Galasso, Gentile et al. 2019). Specifically, fucoxanthin displays anticancer activity in several experimental models against a variety of cancer types including colon cancer (HT-29 cells, 15 µM of fucoxanthin) and leukaemia (HD-60 cells, 10 to 45 µM of fucoxanthin) (Takahashi, Hosokawa et al. 2015). Although the biomass from *Chlorella* and *Arthrospira* are easier to harvest, high-value bioactives such as protein, astaxanthin, $n-3$ PUFA and β-carotene have much higher economic value, even with lower production volumes (Barkia, Saari et al. 2019).

Microalgae have a role in water treatment as they are environmentally friendly and cost-effective (Liu, Saydah et al. 2013). For example, brewery industries, an important part of the producing country's economy, consume large volumes of water and release about 70% of it as wastewater (Amenorfenyo, Huang et al. 2019). Microalage are important agents due to their ability to absorb nutrients and convert them to biomass, for example nitrogen and phosphorus contents in the algal biomass were increased after growing in wastewaters (Amenorfenyo, Huang et al. 2019). Additionally, algae have been used as a green technology for removal of heavy metals from wastewater (Salama, Roh et al. 2019). Microalgae are an emerging source of polysaccharides with actions as prebiotics and probiotics on the gut microbiota, an area of therapeutic interest because of the association with microbial dysbiosis including obesity, inflammatory bowel disease, diabetes, autoimmune and allergic diseases (de Jesus Raposo, de Morais et al. 2016). Seaweeds are an underexploited crop containing bioactive polysaccharides with prebiotic and probiotic actions (Cherry, Yadav et al. 2019). For example, *A. platensis* acted as a probiotic to promote the growth of beneficial bacteria, such as *Lactobacillus casei*, *Streptococcus thermophilus*, and *L. acidophilus* (Parada, de Caire et al. 1998, Bhowmik, Dubey et al. 2009). The vast majority of probiotics are generally regarded as safe and beneficial for healthy individuals; although in patients with compromised immune systems, caution is needed in selecting and administering probiotics because commonly observed adverse effects include sepsis and gastrointestinal ischaemia (Fijan 2014).

Another application of algal polysacchairdes has been for cosmetics because of their skin-protective effects, including anti-wrinkle, lightening, moisturising, UV

protective, anti-oxidative and anti-inflammatory activities (Priyan Shanura Fernando, Kim et al. 2018. doi: 10.1080/07388551.2018.1503995). In particular, the skin-protective effects of carotenoids are attributed to their accumulation mainly into the epidermis after gut absoprtion (Balic and Mokos 2019). Other biological characteristics of sulphated polysaccharides include anti-oxidant, anti-inflammatory, anticoagulant and immunomodulatory properties (Wang, Wang et al. 2014).

Biotechnology application of curcumin

Curcumin is the major active constituent of turmeric, isolated from the rhizomes of *Curcuma longa*. Turmeric contains hundreds of potentially bioactive compounds with curcumin present at 0.4%‑7.2% of dry weight (Akbar, Kuanar et al. 2016). Turmeric is commonly used as a spice and food-colouring agent while curcumin has been proposed for the management of a wide range of diseases including metabolic syndrome, arthritis, anxiety and hyperlipidaemia (Hewlings and Kalman 2017). However, curcumin has very low oral bioavailability in rats, due to its hydrophobic nature, rapid metabolism and systemic elimination.

The therapeutic usefulness of curcumin has been strongly questioned because of its interference with many assays and producing misleading and false results leading to the description of curcumin as an unstable, reactive and non-bioavailable compound, and to its classification as a pan-assay interference compound and an invalid metabolic panacea candidate (Nelson, Dahlin et al. 2017). Therefore, that review concluded that curcumin does not warrant further investigation as a therapeutic agent (Nelson, Dahlin et al. 2017). However, this is an opposing view of many clinical trials that have shown safety, tolerability and effectiveness of curcumin against chronic diseases in humans (Shishodia 2013, Hewlings and Kalman 2017, Sundar Dhilip Kumar, Houreld et al. 2018, Salehi, Stojanovic-Radic et al. 2019).

Nanoparticle preparations of curcumin encapsulated in poly (lactic–co–glycolic acid) (PLGA) increased bioavailability by ~9-fold more than co-administration with piperine which was 3-fold more than unformulated curcumin (Shaikh, Ankola et al. 2009). Curcumin nanoformulations increased solubilisation of curcumin and protected curcumin against inactivation by hydrolysis (Liu, Zhai et al. 2016). Curcumin nanoparticle formulations have increased bioavailability and hence may allow low-dose formulations of food-derived compounds such as curcumin to attenuate chronic systemic disease despite intrinsically low oral bioavailability.

Metabolic syndrome components and treatment

Cardiovascular disease and type 2 diabetes are major causes of morbidity and mortality worldwide. Individuals with metabolic syndrome are more likely to develop cardiovascular disease and type 2 diabetes. Metabolic syndrome is defined as a group of physiological and biochemical factors that increase the risk of cardiovascular disease and type 2 diabetes (O'Neill and O'Driscoll 2015). There are slightly different reference values used to define metabolic syndrome. Metabolic syndrome is defined as a cluster of at least three out of five clinical risk factors: abdominal (visceral) obesity, hypertension, elevated serum triglycerides, low serum high-density lipoprotein and insulin resistance (Alberti, Eckel et al. 2009).

Obesity is the accumulation of excess fat; for adults, a body mass index (BMI) of 25-30 kg/m^2 is defined as overweight and a BMI of 30 kg/m^2 or higher is defined as obese (Apovian 2016). For every 5-unit increase in BMI over 25 kg/m^2, overall mortality increases by 29%, vascular mortality by 41% and diabetes related mortality by 210% (Whitlock G, Lewington S et al. 2009). During 2017-18 in Australia, 67% of adults were overweight or obese, an increase from 63.4% in 2014-15 (Australian Bureau of Statistics). Currently, worldwide metabolic syndrome prevalence is approximately 24% depending on the definition used. This places a large burden on healthcare systems, increasing personal cost and reducing patients' quality and quantity of life. Increasing obesity rates coincides with increasing metabolic syndrome prevalence (Ford, Giles et al. 2002). Obesity affects people from different socioeconomic standings, ethnicities and ages (O'Neill and O'Driscoll 2015).

Obesity is characterised by low-grade chronic inflammation in all tissues (Castro, Macedo-de la Concha et al. 2017). In obesity-related pro-inflammatory states, the increased size of adipocytes leads to an increased production of adipocytokines which initiates a series of inflammation-related pathophysiological processes (Van Gaal, Mertens et al. 2006). Increased triglycerides and low-density lipoprotein cholesterol with decreased high-density lipoprotein cholesterol are linked to an increased risk of cardiovascular disease (Yokoyama, Tani et al. 2019). Insulin resistance is often clustered with lipid abnormalities, glucose intolerance and hypertension (Van Gaal, Mertens et al. 2006). In people with normal insulin sensitivity, lipolysis of adipose tissue is well-regulated with the release of non-esterified fatty acids (NEFA) relative to the energy requirement of the different tissues

(Van Gaal, Mertens et al. 2006). However, in people with insulin resistance, the hypertrophic adipocytes lead to increased lipolysis and release of NEFA (Van Gaal, Mertens et al. 2006).

Hypertension, defined as a systolic blood pressure ≥140 mmHg and/or a diastolic blood pressure ≥90 mmHg, is one of the most common chronic diseases. In 2010, 31.1% of the world's adults were diagnosed with hypertension, although hypertension is the leading preventable cause of premature death worldwide (Mills, Bundy et al. 2016). Treatment and control of hypertension are critically important for the prevention of consequent cardiovascular and kidney diseases (Pereira, Lunet et al. 2009). NAFLD refers to the accumulation of hepatic steatosis not due to excess alcohol consumption and its pathogenesis is related to obesity and insulin resistance. The prevalence of NAFLD is up to 30% in developed countries and approximately 10% in developing countries, therefore it is the most common liver condition in the world (Smith and Adams 2011).

There is no pharmacological intervention for metabolic syndrome, although each risk factor can be addressed individually. For example, treatments include statins for dyslipidaemia, renin-angiotensin-aldosterone system inhibitors for arterial hypertension, metformin for glucose intolerance and glucagon-like peptide 1 receptor agonists to decrease body weight and waist circumference (Rask Larsen, Dima et al. 2018). However, the continuing increase in the prevalence of metabolic syndrome suggests that current treatment options are insufficient. Functional foods have little or no adverse effects and can be used to prevent or reverse some disease states whereas drugs have adverse effects which resulted in the withdrawal of 25 anti-obesity medications between 1964 and 2009 (Onakpoya, Heneghan et al. 2016). Since 1980, the prevalence of obesity has doubled in more than 70 countries and has continuously increased in most other countries (Afshin, Forouzanfar et al. 2017). In 2015, 107.7 million children and 603.7 million adults were obese. The incidence of metabolic syndrome often follows the incidence of obesity and type 2 diabetes, both of which have increased (Saklayen 2018).

There is strong evidence which supports a healthy diet and daily exercise as keys in maintaining health (Warburton, Nicol et al. 2006), for example, the role of the Mediterranean diet in preventing diabetes and metabolic syndrome (Panagiotakos and Polychronopoulos 2005) and exercise in regulating energy intake and expenditure (Chaput, Klingenberg et al. 2011). The Asia-Pacific region has half the world's

population and has seen a rapid increase of prevalence of obesity, type 2 diabetes and cardiovascular disease (Ranasinghe, Mathangasinghe et al. 2017). One fifth of adults in Asia-Pacific countries have metabolic syndrome (Ranasinghe, Mathangasinghe et al. 2017).

Rodents are widely used to mimic human diseases to improve understanding of the causes and progression of disease symptoms and to test potential therapeutic interventions. Research in rodent models that closely mimic the changes in humans is essential. The key measure of the effectiveness of a rodent model is whether it imitates the metabolic syndrome symptoms, especially obesity, diabetes, hypertension and dysfunction of the heart, blood vessels, liver and kidney. Further, as diet is the primary factor responsible for developing metabolic syndrome in humans, the rodent models must be diet-induced to be representative of the real world situation. The model that comes closest to fulfilling this is the high-carbohydrate, high-fat diet-fed male rodent (Panchal and Brown 2011). Therefore, to investigate the potential functional food capabilities of macroalgae, microalgae and curcumin nanoparticles, the current thesis has used a validated model which, in comparison with other models of diabetes and obesity, more closely mimics the changes observed in human metabolic syndrome (Panchal, Poudyal et al. 2011).

Gut microbiota

The human gastrointestinal tract is colonised by up to 100 trillion microbes including bacteria, archaea, viruses, fungi and protozoa, collectively termed the gut microbiota (Qin, Li et al. 2010). It has been recognised to have key roles in health and disease (Thursby and Juge 2017). The beneficial role of the gut microbiota include maintenance of physiological homeostasis. The dysregulation, termed dysbiosis, alters gut bacteria content and can modify intestinal permeability triggering inflammation. Thus, dysbiosis has been implicated in metabolic disorders including obesity, insulin resistance and the inflammatory process (Marcelino, Hiane et al. 2019). It is widely accepted that obesity and associated metabolic diseases, including type 2 diabetes, are closely linked to diet. The Western diet has profound effects on the diversity and populations of microbial species that make up gut microbiota. There are clear links emerging between the microbiome and its effects on the host. In a healthy diet, the bacterial fermentation of fibre into SCFA promotes microbial diversity and is one mechanism by which high fibre intake inhibits weight gain. An increased ratio of

Firmicutes to *Bacteroidetes* is an indicator of dysbiosis and also obesity (Koliada, Syzenko et al. 2017).

Red, green and brown marine seaweeds have shown antimicrobial actions (Pérez, Falqué et al. 2016) and therefore may be used to modulate gut microbiota composition to improve health. In a study, 80 seaweed species were screened against bacterial and fungal pathogens, of which 70% exhibited antibacterial activity but only 27.5% showed antifungal activity (Padmakumar and Ayyakkannu 1997). Among the species tested, *Staphylococcus aureus*, *Vibrio* species and *Trichophyton mentagrophytes* were the most susceptible, whereas *Pseudomonas aeruginosa* and *Aspergillus flavus* were the most resistant (Padmakumar and Ayyakkannu 1997). However, the gut microbiota has also become a focus for research at the intersection of diet and metabolic health (Sonnenburg and Backhed 2016). Recently, because of high-throughput sequencing technologies and associated developments in big-data bioinformatics, strong relationships between gut microbiota and a broad spectrum of diseases including metabolic syndrome have been made (Chassaing, Koren et al. 2015).

Lipopolysaccharides (LPS) are a major component of gram-negative bacteria and cause endotoxaemia which activates gram-negative bacteria and toll-like receptor 4 which regulates pro-inflammatory cytokines (Rogero and Calder 2018). Increased LPS in the bloodsteam is related to high consumption of saturated fats and this stimulates endotoxaemia and inflammatory cascades (Rogero and Calder 2018). LPS has been been extensively studied as it is one of the pathogen-associated molecular patterns which drives an immune response and results in systemic and tissue-specific inflammation and are implicated in cardiovascular risk (Mogensen 2009). For example, in one study, the concentration of circulating endotoxins was highest in patients with the highest cardiovascular burden (McIntyre, Harrison et al. 2011). Gut dysbiosis suppressed the expression of tight junction proteins (Cani, Amar et al. 2007), leading to an increase in intestinal permeability and subsequently the translocation of LPS into the blood (Harris, Kassis et al. 2012).

The consumption of seaweeds may improve the composition of the gut microbiota. For example, the use of seaweed-derived polysaccharides as prebiotics which are fermented enable selective gut commensal metabolism (Cherry, Yadav et al. 2019). Humans lack the ability to digest $\beta(1\rightarrow4)$ linkages in glucan polysaccharides, as in cellulose and hemicelluloses, such as xyloglucan and this

indigestible material is referred to as dietary fibre (Lovegrove, Edwards et al. 2017). In humans, only starch and simple sugars such as glucose, sucrose and lactose can be processed by enzymes encoded by the human genome. Hence, most of the polysaccharides transit the gastrointestinal tract and reach the distal gut where it is subjected to fermentation and becomes the food for the commensal bacteria (Grundy, Edwards et al. 2016). The end-products are termed SCFA and have a physiological function. The undigested materials continue on to the large intestine where microbial co-metabolism ferments substrates such as non-starch polysaccharides, resistant starch and oligosaccharides to produce SCFA (Wells, Potin et al. 2017).

The gut microbiome is implicated in NAFLD with a prevalence of 6-35% in Western countries (Liu, Liu et al. 2019). The gut microbiome has been identified in the past decade as an important factor involved in obesity. An obese microbiome has an increased capacity to harvest energy from the diet (Turnbaugh, Ley et al. 2006). Fermented fibre from marine sources may help modulate the gut microbiome (Shang, Jiang et al. 2018). Marine polysaccharides contain a class of dietary fibre which reach the distal gut and are fermented by gut microbiota and thus exert a fundamental impact on intestinal ecology (Shang, Jiang et al. 2018).

Prebiotics

Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health (Gibson and Roberfroid 1995). The following criteria are used to classify a compound as a prebiotic: (i) it is resistant to acidic pH of stomach, cannot be hydrolysed by mammalian enzymes, and also should not be absorbed in the gastrointestinal tract, (ii) it can be fermented by intestinal microbiota, and (iii) the growth and/or activity of the intestinal bacteria are selectively stimulated by this compound and this process improves host's health (Gibson, Scott et al. 2010). SCFA are the products of prebiotic degradation by anaerobic intestinal microbiota and are small enough molecules to diffuse through gut enterocytes and enter the blood circulation. Therefore, prebiotics are able to affect the gastrointestinal tract and other distant site organs and systems (den Besten, van Eunen et al. 2013). A main mechanism of action of prebiotics is the ability to decrease gut pH because the fermentation products are weak acids (Davani-Davari, Negahdaripour et al. 2019).

Thesis objectives and hypotheses

There is supporting evidence in the literature that seaweeds and microalgae have several bioactive compounds with therapeutic potential symptoms of metabolic syndrome. The objectives of my thesis are to determine whether selected macroalgae and microalgae can prevent or attenuate symptoms of metabolic syndrome. Additionally, due to the recent relevations regarding the role of gut microbiome in numerous diseases, this thesis will aim to correlate physiological effects to gut microbiome changes. I hypothesise that in Chapter 2, 5% *K. alvarezii* will prevent symptoms of metabolic syndrome in rats. In Chapters 3-6, I hypothesise that 5% *S. filiforme, S. siliquosum, C. lentillifera* or *N. oceanica*, respectively, will attenuate symptoms of diet-induced metabolic syndrome in rats. In particular, physiological and metabolic parameters including body weight loss, decreased systolic blood pressure, improved lipid profile, decreased infiltration of inflammatory cells and decreased left ventricular diastolic stiffness will be observed. The mechanism of action will involve both the sulphated and non-sulphated polysaccharides derived from macroalgae exerting prebiotic effects and modulating the gut microbiome comparable to the control rats without metabolic syndrome. In Chapter 7, I hypothesise that a curcumin nanoparticle formulation will increase the low bioavailability of curcumin. Therefore, in the future, aspects of seaweeds that have an inherent barrier such as low bioavailability of carotenoids may be overcome by using biotechnology to unlock the full potential of natural products as functional foods against metabolic syndrome.

CHAPTER 2. κ-CARRAGEENAN IN DIET-INDUCED METABOLIC SYNDROME

The many studies linking the intake of seaweeds with improved human health have been summarised in Chapter 1 of this thesis. Seaweeds have been divided into three main groups: red with more than 7200 species, green with about 2000 species and brown with more than 1800 species (http://www.seaweed.ie/). The red seaweed, *Kappaphycus alvarezii*, contains κ-carrageenan which is a linear polysaccharide with one sulphate per disaccharide unit. It is a soluble fibre with reported cardiovascular and metabolic health benefits. *K. alvarezii* is the main source of κ-carrageenan, a commercial thickening and gelling agent.

This chapter evaluated the potential of *K. alvarezii* to prevent metabolic syndrome developing in rats fed a high-carbohydrate, high-fat diet lnown to produce the cardiovascular and metabolic signs that characterise metabolic syndrome in humans. My hypothesis is that consumption of *K. alvarezii* as a whole seaweed supplement may prevent these abnormalities. The successful outcome may be due to a mechanism that involves κ-carrageenan acting as a prebiotic that modulates the gut microbiome. The prevalance of metabolic syndrome in many Western countries, including Australia and the USA, is remarkably high; attempts to reverse metabolic syndrome through the use of pharmacotherapy have not decreased the incidence of metabolic syndrome in these countries. However, in south-east Asian countries such as China, Japan and Korea, the prevalence of metabolic syndrome is lower, possibly due to their diet which contains seaweed. Hence, the potential impact of these findings relate to an increased use of food supplements to prevent metabolic syndrome.

Article

Kappaphycus alvarezii as a Food Supplement Prevents Diet-Induced Metabolic Syndrome in Rats

Stephen Wanyonyi [1], **Ryan du Preez** [1,2], **Lindsay Brown** [1,2], **Nicholas A. Paul** [3] [ID] **and Sunil K. Panchal** [1,*] [ID]

1   Functional Foods Research Group, Institute for Agriculture and the Environment,
    University of Southern Queensland, Toowoomba, QLD 4350, Australia;
    Stephen.Wanyonyi@usq.edu.au (S.W.); Ryan.duPreez@usq.edu.au (R.d.P.); Lindsay.Brown@usq.edu.au (L.B.)
2   School of Health and Wellbeing, University of Southern Queensland, Toowoomba, QLD 4350, Australia
3   Faculty of Science, Health, Education and Engineering, University of the Sunshine Coast,
    Maroochydore, QLD 4558, Australia; npaul@usc.edu.au
*   Correspondence: Sunil.Panchal@usq.edu.au; Tel.: +61-7-4631-1012

Abstract: The red seaweed, *Kappaphycus alvarezii*, was evaluated for its potential to prevent signs of metabolic syndrome through use as a whole food supplement. Major biochemical components of dried *Kappaphycus* are carrageenan (soluble fiber ~34.6%) and salt (predominantly potassium (K) 20%) with a low overall energy content for whole seaweed. Eight to nine week old male Wistar rats were randomly divided into three groups and fed for 8 weeks on a corn starch diet, a high-carbohydrate, high-fat (H) diet, alone or supplemented with a 5% (*w/w*) dried and milled *Kappaphycus* blended into the base diet. H-fed rats showed symptoms of metabolic syndrome including increased body weight, total fat mass, systolic blood pressure, left ventricular collagen deposition, plasma triglycerides, and plasma non-esterified fatty acids along with fatty liver. Relative to these obese rats, *Kappaphycus*-treated rats showed normalized body weight and adiposity, lower systolic blood pressure, improved heart and liver structure, and lower plasma lipids, even in presence of H diet. *Kappaphycus* modulated the balance between Firmicutes and Bacteroidetes in the gut, which could serve as the potential mechanism for improved metabolic variables; this was accompanied by no damage to the gut structure. Thus, whole *Kappaphycus* improved cardiovascular, liver, and metabolic parameters in obese rats.

Keywords: metabolic syndrome; red seaweed; obesity; inflammation; carrageenan; *Kappaphycus alvarezii*; potassium; salt

1. Introduction

Regular consumption of seaweeds in Japan, Korea, and China occurs together with a relatively low incidence of cardiovascular and metabolic disorders relative to Western countries [1–4]. In Japan, for example, more than 20 species of seaweeds are regularly included in meals and each have different biochemical properties and herbal uses [5]. This diversity of seaweeds in the diet suggests that supplementation of Western diets with seaweeds should be investigated as an intervention to reduce the incidence of cardiovascular disease and diabetes.

Edible seaweeds contain a large variety of phylum-specific dietary fiber including alginates, fucans, and laminarans from brown seaweeds (Phylum Ochrophyta, Class Phaeophyceae), galactans, agar, and carrageenans from red seaweeds (Phylum Rhodophyta), and ulvans from green seaweeds (Phylum Chlorophyta) [6–8]. A modest 8 g/day serving of seaweed could provide up to 12.5% of the recommended daily intake of dietary fiber [9]. Additionally, seaweeds contain varying amounts of protein, with some red seaweed species containing up to 26.6% proteins [10], so that dietary seaweeds

confer the advantage of low energy density [9]. Most of the seaweeds that are staple foods in Japan, China, and Korea are cold-water genera of brown (*Saccharina/Laminaria*—kombu, *Undaria*—wakame, *Sargassum*—hijiki) or red seaweeds (*Porphyra/Pyropia* species—nori). These species have dominated the anti-obesity studies to date, with far less known about the main species of tropical red seaweeds that are now being cultured on a large scale in Indonesia and the Philippines. A recent study with one such seaweed, *Gracilaria*, revealed its potential as an intervention against metabolic diseases [11]. In streptozotocin-induced diabetic mice, polysaccharides from *Gracilaria lemaneiformis* were more effective at regulating the blood insulin concentrations, lipid parameters, and blood urea nitrogen than metformin [11]. However, our understanding of the potential of red seaweed commodities including *Kappaphycus alvarezii* (hereafter *Kappaphycus*) as functional foods remains limited.

Functional foods such as red seaweeds could serve as potential therapeutic options for metabolic syndrome [12], a constellation of risk factors for type 2 diabetes and cardiovascular disease. There is some evidence for antioxidant and blood lipid-lowering properties of *Kappaphycus* [13,14]. Furthermore, an in vitro study using the extracted sulfated fractions of κ-carrageenan from *Kappaphycus* demonstrated the potential of this seaweed as an intervention for colon cancer [15]. However, the major problem with the studies to date on *Kappaphycus* is that the focus has been almost exclusively on the use of extracted carrageenan for its effects. Thus, this study looked at the whole *Kappaphycus* as the intervention, acknowledging that the dried seaweed product is more than a source of carrageenans, and the investigation of this functional food requires use of the whole seaweed. Further, isolated carrageenan has been used to induce paw edema to test anti-inflammatory compounds [16–18], which is contradictory to the hypothesis of this study. As carrageenan is a soluble fiber, it has potential to slow down digestion [9] and hence help in improving metabolism.

In this study, we have evaluated the potential of *Kappaphycus* as a whole food to attenuate the development of obesity in high-carbohydrate, high-fat diet-fed rats that mimic symptoms of human metabolic syndrome including central obesity, hypertension, dyslipidemia, and impaired glucose tolerance together with the cardiovascular and liver complications of metabolic syndrome [19]. We evaluated the responses to *Kappaphycus* in a prevention protocol where rats were given high-carbohydrate, high-fat diet with *Kappaphycus* for a total of 8 weeks. Additionally, we investigated the effect of supplementation with *Kappaphycus* on the balance of gut microbiota as a potential mechanism for improving metabolic syndrome.

2. Materials and Methods

2.1. Red Seaweed Source

Air-dried samples of *Kappaphycus* were collected from a seaweed trader in Suva, Fiji, in June 2015. The source of *Kappaphycus* was shallow water farms located at Yaqeta village in the Yasawa Islands, Fiji. Two bulk samples of ~3 kg each, comprising multiple individual thalli, were packed in vacuum-sealed bags containing silica desiccant and transported to Australia.

2.2. Seaweed Analysis

The two 3 kg samples of *Kappaphycus* were dried to <2% moisture content and subsequently milled using a blender prior to further analyses. A total of 1 kg of each replicate sample was combined, homogenized, and stored at −20 °C prior to use in the rat study. The biochemical profile of each 3 kg milled seaweed sample was analyzed separately for its proximate composition (dietary fiber, protein, lipid, ash, and energy content) and elemental composition (carbon (C), hydrogen (H), oxygen (O), nitrogen (N), sulfur (S), metals, metalloids, and halogens).

For the proximate analyses, total dietary fiber content, including soluble and insoluble components, was analyzed by Grain Growers Ltd. (Sydney, NSW, Australia) on a 10 g sub-sample following standard methods (Association of Official Analytical Chemists (AOAC) Official Method 985.29 total dietary fiber in foods, and AOAC Official Method 993.19 soluble dietary fiber in food and

food products). Protein content was determined as the sum of amino acids based on quantitative amino acid analysis performed at The Australian Proteome Analysis Facility [20]. Ash content was quantified by combustion in air (550 °C, 6 h) (SEM muffle furnace, LabTek, Brendale, QLD, Australia). Lipid content was quantified on a 200 mg sub-sample using solvent extraction [21,22]. Moisture content was measured on a 1 g biomass at 105 °C to constant weight (MS-70 moisture analyzer, A & D Company Ltd., Thebarton, SA, Australia). Carbohydrates were calculated by difference as $100 - \Sigma$ (lipid, protein, ash, moisture) where lipid, protein, ash, and moisture contents were expressed as a percentage of the original biomass. The higher heating value (HHV) or energy content (kJ/g) of *Kappaphycus* was calculated based on ash content and C, H, O, N and S elemental composition [23].

A sub-sample (200 mg) of each replicate was analyzed for the content of C, H, O, N, and S as well as Cl, Br, F, and I (OEA Laboratory Ltd., Cornwall, UK). The content of metals and metalloids (22 elements) of the seaweed was measured by Inductively Coupled Plasma Mass Spectrometry (ICP/MS with Varian 820-MS, Mulgrave, VIC, Australia) at the Advanced Analytical Centre of James Cook University, Townsville, Australia.

2.3. Rats and Diets

All experimental protocols were approved by the Animal Ethics Committee of the University of Southern Queensland (approval number 15REA007) under the guidelines of the National Health and Medical Research Council of Australia. The experimental protocol consisted of 30 male Wistar rats (8–9 weeks old; 330–335 g) purchased from the Animal Resource Centre, Murdoch, WA, Australia. Rats were randomly divided into three experimental groups each consisting of ten rats. One group was fed on corn starch diet (C) which did not induce any complications of metabolic syndrome. The second group was fed with a high-carbohydrate, high-fat diet (H) to induce metabolic syndrome. The third group was fed with high-carbohydrate, high-fat diet supplemented with dried milled *Kappaphycus* (HR, 5% of the food). All groups were fed for 8 weeks on their respective diets.

All rats were housed in individual cages in a temperature-controlled (21 ± 2 °C) room with an automated 12-h light/dark cycle environment and *ad libitum* access to food and water. C diet was prepared by thoroughly mixing 570 g corn starch, 155 g powdered rat food, 25 g Hubbel, Mendel, and Wakeman salt mixture (MP Biomedicals, Seven Hills, NSW, Australia), and 250 g water per kilogram of food. H diet was prepared by thoroughly mixing 175 g fructose, 395 g condensed milk, 200 g beef tallow, 155 g powdered rat food, 25 g Hubbel, Mendel, and Wakeman salt mixture, and 50 g water per kilogram of food [19]. HR diet was prepared by replacing water with dried milled *Kappaphycus*. C rats were given normal drinking water whereas H and HR rats were given 25% fructose (w/v) in drinking water. The energy content of the diet and feed conversion efficiency were calculated [19]. Rats were measured daily for their body weights and intakes of food and water.

2.4. Systolic Blood Pressure

Systolic blood pressure was measured under light sedation by intraperitoneal injection with Zoletil (tiletamine 10 mg/kg, zolazepam 10 mg/kg; Virbac, Peakhurst, NSW, Australia) [19].

2.5. Oral Glucose Tolerance Test

Oral glucose tolerance test was performed on rats after overnight (~12 h) food deprivation [19]. Briefly, basal blood glucose concentrations were determined in tail vein blood using Medisense Precision Q.I.D. glucometer (Abbott Laboratories, Bedford, MA, USA). After the initial blood glucose measurements, rats were given 2 g/kg body weight of glucose as a 40% (w/v) aqueous glucose solution via oral gavage. Tail vein blood samples were then taken 30, 60, 90, and 120 min after glucose administration [19]. During the food-deprivation period, fructose-supplemented drinking water in H and HR rats was replaced with normal drinking water.

2.6. Body Composition

Dual-energy *X*-ray absorptiometry was performed on all rats at the end of protocol using a Norland XR36 DXA instrument (Norland Corp., Fort Atkinson, WI, USA) [19]. Briefly, rats were anesthetized by intraperitoneal injection of Zoletil (tiletamine 10 mg/kg and zolazepam 10 mg/kg) and Ilium Xylazil (xylazine 6 mg/kg; Troy Laboratories, Smithfield, NSW, Australia) [19]. Visceral adiposity index (%) was calculated [24].

2.7. Organ Weights

Terminal euthanasia was induced by intraperitoneal injection of Lethabarb (pentobarbitone sodium, 100 mg/kg; Virbac, Peakhurst, NSW, Australia) and ~6 mL blood was immediately drawn from the abdominal aorta and processed for plasma collection [19]. Hearts were separated into right ventricle and left ventricle with septum for weighing. Livers and abdominal fat pads (retroperitoneal, epididymal, and omental) were isolated and weighed. Organ weights were normalized to the tibial length at the time of isolation and the final organ weights were presented in mg of tissue/mm of tibial length [19].

2.8. Histology

Hearts and livers from rats in each group were collected and fixed in 10% neutral buffered formalin for 3 days. Tissues were then processed and blocked in wax before thin sections (5 μm) were cut and placed on slides. Heart tissues were stained with hematoxylin and eosin or picrosirius red. Liver, ileum, and colon sections were stained with hematoxylin and eosin. Images were taken using an EVOS FL Colour Imaging System (v 1.4 (Rev 26059); Advanced Microscopy Group, Bothell, WA, USA).

2.9. Biochemical Analyses of Rat Plasma

Plasma activities of alanine transaminase and aspartate transaminase, and plasma concentrations of total cholesterol, triglycerides, non-esterified fatty acids, and potassium (K) were determined [19].

2.10. Fecal Lipid Measurements

Fecal lipids were extracted using Folch method [21]. Briefly, 1 g of freshly collected feces were air dried and added to a homogenization tube containing 5 mL saline. The pellets were homogenized to form a suspension. The suspension was thoroughly mixed with an equal volume of chloroform in methanol (2:1, *v/v*) and centrifuged at $1000\times$ g for 10 min at room temperature. After centrifugation, the lower liquid phase was isolated and transferred to a separate vial. The extracted lipids were air-dried and weighed.

2.11. Gut Microbiota Diversity Profiling

Immediately following euthanasia and organ removal, two or three fecal pellets were collected from the colon of rats and stored at −80 °C in nuclease-free tubes. DNA extraction and diversity profiling were performed by the Australian Genome Research Facility, Brisbane, QLD, Australia. The V3-V4 region of the 16S rRNA gene was selected for amplification. The primers used were F341 (5′-CCTAYGGGRBGCASCAG-3′) and R806 (5′-GGACTACNNGGGTATCTAAT-3′). PCR amplicons were generated using AmpliTaq Gold 360 mastermix (Life Technologies, Scoresby, VIC, Australia) for the primary PCR. A secondary PCR to index the amplicons was performed with TaKaRa Taq DNA Polymerase (Clontech, Mountain View, CA, USA). The resulting amplicons were measured by fluorometry (Invitrogen Picogreen, Mount Waverley, VIC, Australia) and normalized. The equimolar pool was then measured by qPCR (KAPA) followed by sequencing on the Illumina MiSeq (Illumina Inc., San Diego, CA, USA) with 2×300 base pairs paired-end chemistry.

Paired-ends reads were assembled by aligning the forward and reverse reads using PEAR (version 0.9.5, The Exelixis Lab, Heidelberg, Germany) [25]. Primers were identified and trimmed.

Trimmed sequences were processed using Quantitative Insights into Microbial Ecology (QIIME 1.8) [26] USEARCH (version 7.1.1090) [27,28] and UPARSE software [29]. Using USEARCH, sequences were quality filtered, full length duplicate sequences were removed and sorted by abundance. Singletons or unique reads in the data set were discarded. Sequences were clustered followed by chimera filtered using "rdp_gold" database as the reference. To obtain the number of reads in each Operational Taxonomic Unit (OTU), reads were mapped back to OTUs with a minimum identity of 97%. Using QIIME, taxonomy was assigned using Greengenes database (version 13_8, August 2013) [30]. A heat map was constructed using R statistical software according to the developer's instructions to visualize the relative abundance of each bacterial species and their respective phyla.

2.12. Metal and Metalloid Liver Analyses

Livers were dissected out and dried at 60 °C for 24 h. A suite of 22 elements was analyzed by the Advanced Analytical Centre at James Cook University using Inductively Coupled Plasma Mass Spectrometer.

2.13. Statistical Analysis

All data are presented as mean $\pm$ standard error of the mean (SEM). Differences between the groups were determined by one-way analysis of variance. Statistically significant variables were treated with Neumann-Keuls *post hoc* test to compare all groups of rats. Statistical analyses were performed using GraphPad Prism version 5 for Windows (San Diego, CA, USA). *P* values of <0.05 were considered statistically significant.

3. Results

3.1. Composition of Kappaphycus

Dried *Kappaphycus* contained carbohydrates (38.3% dry weight (d.w.)) and minerals (ash content: 58.0% d.w.) with relatively small amount of proteins (1.34% d.w.; sum of amino acids, Supplementary Table S1) and lipids (0.62% d.w.) and a low energy content of 5.23 kJ/g. The carbohydrate component of the seaweed was further characterized into insoluble and soluble dietary fiber, of which the soluble fiber component comprised 34.6% d.w. of the whole biomass, indicating that 90% of the carbohydrate is soluble fiber.

The mineral content included potassium (K, 20% d.w.) and sodium (Na, 3.7% d.w.), primarily as chloride salts (Cl, 23% d.w.), and the ratio of sodium:potassium (Na:K) was 0.19. There were relatively low concentrations of halogens, for example, bromine (Br) was 0.12% d.w. and iodine (I) was not detected (Supplementary Table S2). A range of other metal and metalloid elements was present, including calcium (Ca, 28.96 g/kg) and magnesium (Mg, 5.69 g/kg), as well as other trace elements including iron (Fe), boron (B), and manganese (Mn) (Supplementary Table S3).

3.2. Diet Intake and Body Composition

At the end of the protocol, body weight and body mass index of H rats were higher than C rats while HR rats had similar body weight and body mass index to C rats (Table 1). Food intake was higher in C rats relative to H and HR rats while energy intake was higher in H and HR rats compared to C rats (Table 1). There were no differences in water intakes between C, H, and HR rats. Feed conversion efficiency was higher in H rats than in C and HR rats (Table 1). H rats had higher abdominal circumference than C rats whereas HR rats had lower abdominal circumference compared to H rats (Table 1). Retroperitoneal, omental, and total abdominal fat were higher in H rats compared to C and HR rats whereas epididymal fat was not different between these groups (Table 1). Similar to total abdominal fat content, total body fat was higher in H rats than in C and HR rats whereas lean mass was similar in C and H rats and lower in HR rats than in C and H rats (Table 1). Bone mineral

content and bone mineral density were higher in H rats compared to C rats while these parameters were lower in HR rats compared to H rats (Table 1).

Table 1. Effects of *Kappaphycus* on metabolic, body composition, and physiological variables.

Variables	C	H	HR
Initial body weight, g	331 ± 1	334 ± 1	332 ± 1
Final body weight, g	350 ± 8 [b]	431 ± 11 [a]	348 ± 6 [b]
Body mass index, g/cm^2	0.57 ± 0.02 [b]	0.70 ± 0.03 [a]	0.58 ± 0.01 [b]
Water intake, mL/day	37.3 ± 7.3	21.7 ± 2.2	35.0 ± 2.6
Food intake, g/day	38.7 ± 3.3 [a]	23.7 ± 2.3 [b]	20.8 ± 2.0 [b]
Energy intake, kJ/day	434 ± 11 [b]	485 ± 8 [a]	507 ± 10 [a]
Feed conversion efficiency, g/kJ	0.04 ± 0.02 [b]	0.20 ± 0.03 [a]	0.03 ± 0.01 [b]
Abdominal circumference, cm	18.4 ± 0.1 [c]	20.5 ± 0.1 [a]	18.9 ± 0.1 [b]
Retroperitoneal fat, mg/mm *	149 ± 14 [b]	284 ± 31 [a]	157 ± 26 [b]
Epididymal fat, mg/mm *	79 ± 10	141 ± 29	81 ± 17
Omental fat, mg/mm *	114 ± 10 [b]	208 ± 20 [a]	99 ± 20 [b]
Total abdominal fat, mg/mm *	342 ± 18 [b]	632 ± 73 [a]	337 ± 48 [b]
Total fat mass, g	49.4 ± 5.0 [b]	98.7 ± 8.3 [a]	53.4 ± 6.5 [b]
Total lean mass, g	315 ± 4 [a]	318 ± 7 [a]	290 ± 6 [b]
Bone mineral content, g	10.1 ± 0.3 [b]	11.8 ± 0.3 [a]	9.8 ± 0.2 [b]
Bone mineral density, g/cm^2	0.165 ± 0.002 [b]	0.180 ± 0.005 [a]	0.156 ± 0.002 [c]
Basal blood glucose concentrations, mmol/L	3.7 ± 0.3 [b]	4.7 ± 0.2 [a]	3.6 ± 0.2 [b]
Area under the curve, mmol/L·min	650 ± 29 [b]	799 ± 27 [a]	753 ± 30 [a]
Total cholesterol, mmol/L	1.50 ± 0.08	1.61 ± 0.09	1.76 ± 0.14
Triglycerides, mmol/L	0.61 ± 0.09 [c]	1.65 ± 0.21 [a]	1.31 ± 0.07 [b]
Non-esterified fatty acids, mmol/L	1.16 ± 0.21 [c]	4.09 ± 0.29 [a]	1.72 ± 0.36 [b]
Fecal lipids, mg/g of feces	0.87 ± 0.04 [b]	1.31 ± 0.04 [a]	0.63 ± 0.03 [c]
Systolic blood pressure, mmHg	120 ± 2 [c]	136 ± 1 [a]	127 ± 3 [b]
LV + septum wet weight, mg/mm *	17.8 ± 0.6	20.0 ± 0.8	19.2 ± 0.9
RV wet weight, mg/mm *	4.25 ± 0.30	4.08 ± 0.46	4.61 ± 0.34
Liver weight, mg/mm *	217 ± 14	286 ± 9	256 ± 30
Plasma ALT activity, U/L	28.0 ± 3.6 [b]	30.1 ± 4.6 [b]	42.0 ± 4.0 [a]
Plasma AST activity, U/L	70.9 ± 3.5	72.1 ± 7.0	74.0 ± 4.0
Plasma potassium, mmol/L	5.7 ± 0.3 [a]	5.0 ± 0.3 [b]	6.1 ± 0.5 [a]

Values are mean $\pm$ SEM, *n* = 8–10. Means in a row with unlike superscripts (a, b, or c) differ and no superscript indicates no significant difference between the groups, *p* < 0.05. ALT, alanine transaminase; AST, aspartate transaminase; C, corn starch diet-fed rats; H, high-carbohydrate, high-fat diet-fed rats; HR, high-carbohydrate, high-fat diet-fed rats supplemented with dried and milled whole *Kappaphycus*. LV, left ventricle; RV, right ventricle. * indicates the values were normalized against tibial length and presented as the tissue weight in mg/mm tibial length.

3.3. Oral Glucose Tolerance, Plasma Biochemistry, and Fecal Lipids

Basal blood glucose concentrations and area under the curve at 8 weeks were higher in H rats compared to C rats (Table 1). Although basal blood glucose concentrations at 8 weeks were lower in HR rats than in H rats, the areas under the curve were similar in H and HR rats (Table 1). Plasma total cholesterol was unchanged between the groups whereas plasma triglycerides and non-esterified fatty acids were in the order of C < HR < H (Table 1). However, total cholesterol concentrations were not different. HR diets decreased plasma non-esterified fatty acids and triglycerides (Table 1). Fecal lipids were higher in H rats compared to C rats where HR had lower fecal lipids than H rats (Table 1). Plasma potassium concentrations were lower in H rats compared to C rats whereas the HR rats had higher plasma potassium concentrations than H rats (Table 1).

3.4. Cardiovascular Structure and Function

At 8 weeks, systolic blood pressure was higher in H rats than C rats. *Kappaphycus* suppressed the increase in blood pressure in HR rats in comparison to H rats; however, the systolic blood pressure was higher in HR rats than C rats. There were no differences in LV + septum wet weight and RV wet weight between the groups (Table 1). H rats had higher infiltration of inflammatory cells and collagen

deposition in the left ventricle than in C rats whereas HR rats had lower infiltration of inflammatory cells and collagen deposition than H rats (Figure 1).
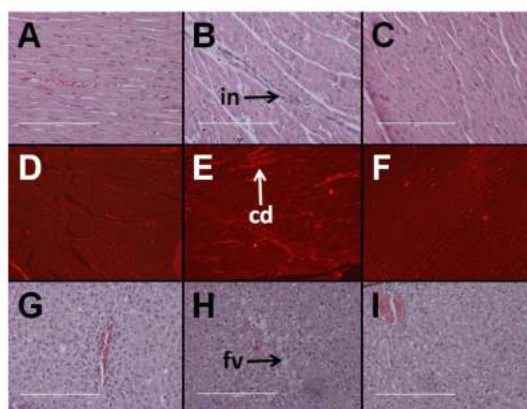

Figure 1. Effects of *Kappaphycus* on inflammation (top row—"in") and collagen deposition (middle row—"cd") in the heart using hematoxylin and eosin stain and picrosirius red stain, respectively, in C rats (**A**,**D**), H rats (**B**,**E**), and HR rats (**C**,**F**). Effects of *Kappaphycus* on inflammation and fat deposition (bottom row—"fv") in the liver using hematoxylin and eosin stain in C rats (**G**), H rats (**H**), and HR rats (**I**).

3.5. Hepatic Structure and Function

Hematoxylin and eosin staining of liver sections from C rats showed that there was minimal fat deposition in the livers of C rats and infiltration by inflammatory cells was not observed (Figure 1G). H rats showed the presence of fat vacuoles and infiltration of inflammatory cells (Figure 1H). HR rats had lower fat deposition in the liver than H rats with very little to no inflammation (Figure 1I). Plasma activities of AST were similar between all the groups. Plasma ALT activities were higher in HR rats than in C and H rats (Table 1).

Metal and metalloid liver analyses revealed widespread decreases in the elemental content of liver of HR rats relative to both C and H rats, with only some minor increases in particular HR vs. C and HR vs. H comparisons (Table 2). As the most prominent examples in comparison to H rats (>1/3 reduction), *Kappaphycus* decreased the concentrations in HR rats of barium (Ba) (to 28% of H level), aluminium (Al) (48%), molybdenum (Mo) (52%), strontium (Sr) (53%), arsenic (As) (62%), and zinc (Zn) (65%). Similarly, in comparison to C rats, arsenic (As) and molybdenum (Mo) decreased to 29% and 64%, respectively, in HR rats.

While there was more potassium (K) in the HR diets (due to the 5% seaweed portion delivering ~10× more potassium (K) to the overall diet), there was a lower concentration of potassium (K) in the liver (HR resulted in 71% of H and HR was 69% of C).

3.6. Gut Structure and Microbiota

Ileum from H rats showed infiltration of inflammatory cells while ileum and colon from C and HR rats along with colon from H rats did not show any structural damages, including no evidence of inflammation. This indicated that the *Kappaphycus* did not cause any inflammatory damage to the gut structure (Figure 2).

In all groups, the major gastrointestinal bacterial phyla, Firmicutes and Bacteroidetes, were predominant (Figure 3). Relative to C and HR rats, H rats had lower abundance of Bacteroidetes by 12.05% and 17.04%, respectively. Conversely, H rats had 26.97% and 19.43% more abundance of Firmicutes relative to C and HR rats, respectively (Figure 3B). There was no significant difference in the abundance of both Firmicutes and Bacteroidetes between C and HR rats. Based on the Shannon diversity index, there was no difference in diversity between the groups (Figure 3C).

Table 2. Effects of *Kappaphycus* on metal and metalloid contents in liver.

Metal (Symbol)	C (in ppm)	H (in ppm)	HR (in ppm)
Aluminium (Al)	5.32 ± 0.89	11.83 ± 2.71	5.71 ± 1.50
Arsenic (As)	4.75 ± 0.71 [a]	2.24 ± 0.34 [b]	1.40 ± 0.16 [b]
Boron (B)	BDL	BDL	BDL
Barium (Ba)	0.07 ± 0.01 [b]	0.23 ± 0.04 [a]	0.07 ± 0.01 [b]
Calcium (Ca)	124 ± 3 [a]	117 ± 6 [a]	94 ± 9 [b]
Cadmium (Cd)	0.06 (*n* =1)	BDL	0.04 ± 0.01 (*n* = 2)
Cobalt (Co)	BDL	BDL	BDL
Chromium (Cr)	BDL	BDL	BDL
Copper (Cu)	13.78 ± 1.32	10.65 ± 0.99	9.95 ± 1.41
Iron (Fe)	575 ± 22 [a]	376 ± 12 [b]	405 ± 38 [b]
Mercury (Hg)	BDL	BDL	BDL
Potassium (K)	10,506 ± 256 [a]	10,146 ± 309 [a]	7225 ± 998 [b]
Magnesium (Mg)	547 ± 10	529 ± 39	434 ± 40
Manganese (Mn)	4.67 ± 0.20	3.92 ± 0.38	4.41 ± 0.49
Molybdenum (Mo)	0.65 ± 0.07 [ab]	0.81 ± 0.05 [a]	0.42 ± 0.09 [b]
Sodium (Na)	1581 ± 97	1,278 ± 44	1060 ± 187
Nickel (Ni)	0.14 (*n* = 1)	BDL	0.57 ± 0.37 (*n* = 4)
Phosphorus (P)	8820 ± 324 [a]	8559 ± 399 [a]	6090 ± 730 [b]
Lead (Pb)	0.15 ± 0.05	0.11 ± 0.02	0.11 ± 0.03
Sulfur (S)	5233 ± 155 [a]	5055 ± 239 [a]	3510 ± 408 [b]
Selenium (Se)	1.54 ± 0.03	1.45 ± 0.13	1.01 ± 0.16
Strontium (Sr)	0.20 ± 0.02 [b]	0.31 ± 0.05 [a]	0.17 ± 0.02 [b]
Vanadium (V)	BDL	BDL	BDL
Zinc (Zn)	63.12 ± 2.81 [a]	66.85 ± 2.36 [a]	43.23 ± 5.13 [b]

Values are mean ± SEM, *n* = 4–5. Means in a row with unlike superscripts (a or b) differ and no superscript indicates no significant difference between the groups, $p < 0.05$. C, corn starch diet-fed rats; H, high-carbohydrate, high-fat diet-fed rats; HR, high-carbohydrate, high-fat diet-fed rats supplemented with dried and milled whole *Kappaphycus*; BDL, below detection limit.
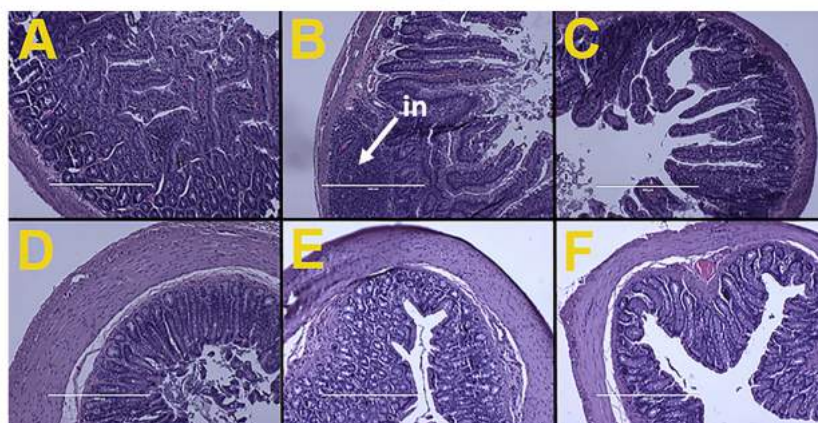
Figure 2. Effects of *Kappaphycus* on structure and inflammation in ileum (top row) and colon (bottom row) using hematoxylin and eosin stain in C rats (**A**,**D**), H rats (**B**,**E**), and HR rats (**C**,**F**). "in"—inflammation.

The relative abundances of all species were compared between the groups to estimate the effect of diet at species level. A cut-off point of 1% abundance in C rats was applied to enhance confidence. The abundance of two species from phylum Bacteroidetes (*Bacteroides* sp., and an unspecified species from the S24-7 family) and 2 species from phylum Firmicutes (*Oscillospira* sp. and an unspecified species from Clostridiaceae family) were differentially affected by diet (Figure 3D). Compared to C rats, there was a 3.5-fold decrease in *Bacteroides* sp. and 4-fold decrease in the S24-7 family species. HR rats had

higher abundance of *Bacteroides* sp. 2.5-fold and 8.8-fold relative to C and H rats, respectively. Similarly, HR rats had higher S24-7 family species by 4.8-fold compared to H rats and normalized its abundance to C rats. H rats had higher abundance of *Oscillospira* sp than C and HR rats (1.7 and 1.9-fold higher, respectively). Similarly, H rats had higher abundance of Clostridiaceae family by 2.8 and 2.7-fold, respectively, relative to C and HR rats. There was no difference in both the Firmicutes species between C and HR rats. Some species in phylum Actinobacteria, for example *Bifidobacterium pseudolongum*, were more abundant in C and HR rats than in H rats, but their abundance was below the cut-off of 1%.
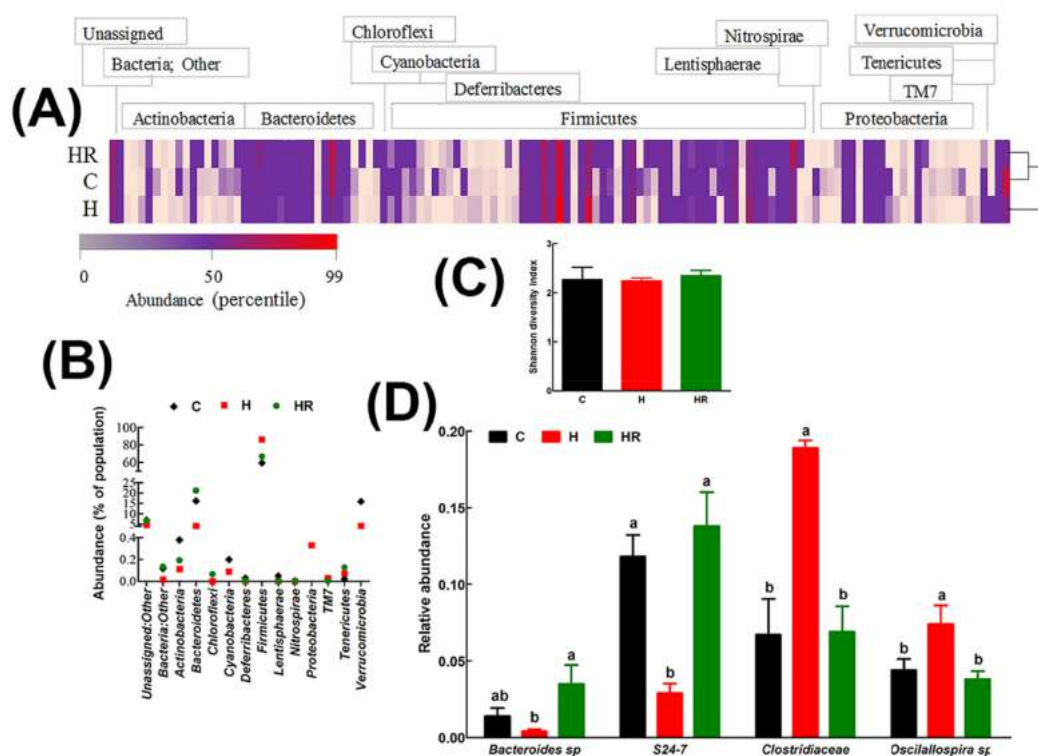
Figure 3. Effect of *Kappaphycus* on gut microbiota diversity profiles. (**A**) Heat map of bacterial species abundance. Abundance values for each species were plotted as a percentile with the most abundant species represented in bright red, the 50th percentile species represented in purple and the lowest value in pink. (**B**) The relative abundance of each phylum presented as a percentage of the total population for each treatment group. (**C**) Shannon diversity index. The index was determined from the means of abundance for each treatment group. (**D**) Relative abundance of species that were differentially regulated by diet. Only species whose mean abundance for the C diet group was equal to or higher than 1% were plotted in order to enhance confidence. C, corn starch diet-fed rats; H, high-carbohydrate, high-fat diet-fed rats; HR, high-carbohydrate, high-fat diet-fed rats supplemented with dried and milled whole *Kappaphycus*.

4. Discussion

We have used an established rat model of human metabolic syndrome to demonstrate the anti-obesity properties of whole *Kappaphycus*. This red seaweed has a high content of κ-carrageenan and a low content of digestible carbohydrates coupled with a high potassium (K) content [31]. Foods with a high soluble fiber content improved symptoms of metabolic syndrome by increasing gastrointestinal viscosity thereby inhibiting intestinal absorption of lipids and carbohydrates [8,32–34]. Sulfated polysaccharides, including carrageenans, alginates, and porphyrans derived from red seaweeds, and fucoidans derived from brown seaweeds, have shown anti-obesity, blood glucose-lowering, and blood lipid-lowering effects in different experimental models [32,35,36]. In a mouse model of obesity, treatment with 1% fucoidan suppressed adiposity by transcriptionally inhibiting the

expression of aP2 and PPAR-γ as well as inhibiting acetyl-CoA carboxylase activity, thereby decreasing fatty acid synthesis [36]. In obese individuals, treatment with sodium alginate from the seaweeds *Laminaria hyperborea* and *Laminaria digitata* (Class Phaeophyceae) in a calorie-restricted diet led to increased loss of body weight by 6.4% in a 12-week trial [37].

In this study, treatment with whole *Kappaphycus* decreased central obesity and lowered plasma triglycerides and non-esterified fatty acids. However, it is unlikely that these observed changes were mediated by decreased carbohydrate and lipid absorption due to gastrointestinal viscosity. Lowered fecal lipids suggests that *Kappaphycus* increased lipid catabolism rather than an inhibition of absorption in the gut. It is also possible that, similar to fucoidan, carrageenan attenuated adiposity and dyslipidemia by inhibiting adipogenesis and fatty acid synthesis or increasing lipase activity [36,38,39]. In 3T3-L1 adipocytes, 200 μg/mL fucoidan increased the expression of hormone-sensitive lipase and its phosphorylated form signifying increased lipolysis. Since the decreased triglycerides in our study were accompanied by decreased non-esterified fatty acids, it is possible that, apart from stimulating lipolysis, *Kappaphycus* stimulated fatty acid oxidation as well. Fucoidans potentially increased fatty acid oxidation through stimulation of AMPK activity [40] and carrageenans may have similar AMPK-stimulating properties.

Kappaphycus, a tropical red seaweed (formerly *Eucheuma cottonii*), is a commercial source of carrageenan, a soluble fiber and sulfated polysaccharide that is extracted and used as an emulsifier in the food industry [41]. In addition to fiber, *Kappaphycus* contains many minerals including potassium (K), calcium (Ca), magnesium (Mg), sodium (Na), copper (Cu) and zinc (Zn) [42]. Typically, production of *Kappaphycus* occurs in shallow water fixed-line culture on the reef flat, followed by air-drying for a few days adjacent to the farm sites. *Kappaphycus* is normally dried to 25–40% moisture content and then transported to trading centres and distributed to processors for extraction of semi-refined carrageenan through a heated alkali process.

Throughout the tropical countries, there is now a push for diversification of the products from *Kappaphycus*, with a particular emphasis on cottage industry use of the whole seaweed as a vegetable and in savory and sweet products. This change from the traditional industrial extraction of carrageenan to use as a whole food for local consumption brings with it questions as to the potential health benefits of the unrefined products of dried seaweed.

In many countries, there is a renewed push for the use of whole foods in particular since metabolic diseases associated with energy-rich diets are a leading cause of public health concern [43]. Increased energy intake without increased energy expenditure leads to development of central obesity, hyperglycemia, dyslipidemia, fatty liver, hypertension, and insulin resistance, collectively known as metabolic syndrome [44]. Functional foods such as red seaweeds could serve as potential therapeutic options for these changes [12] and there is some evidence that *Kappaphycus* has antioxidant and blood lipid-lowering properties [13,14]. Furthermore, an in vitro study using the extracted sulfated fractions of κ-carrageenan from *Kappaphycus* demonstrated the potential of this seaweed as an intervention for colon cancer [15].

Contrary to some reports of adverse gastrointestinal effects of foods containing carrageenans [45], this study did not show changes in the mucosal structure of intestine after feeding *Kappaphycus* to rats. However, the focus to date on the potential effects of carrageenan, both positive and negative, ignores the potential independent and interactive effects of the other main components, in particular the seaweed salt as the major component of *Kappaphycus*.

Phytochemicals from *Kappaphycus* other than carrageenans may confer beneficial effects. For example, phycoerythrin and phycocyanin may stimulate triglyceride catabolism by providing an antioxidant environment [46] which is required for optimal lipase activity [47]. *Kappaphycus* also contains monounsaturated fatty acids and polyunsaturated fatty acids [42,48] which are associated with beneficial changes in metabolic syndrome such as increased HDL-cholesterol, decreased triglycerides, and improved cardiovascular and liver health [49,50]. However, these pigments and fatty acid

components are unlikely to be present in sufficient quantities in *Kappaphycus* to produce these changes in metabolic syndrome [32].

Potassium may be present in sufficient amounts in *Kappaphycus* to produce physiological changes. In this study, a lower serum potassium concentration in obese rats was accompanied by pathological changes in the cardiovascular system. Potassium is a key determinant of cardiovascular health with low dietary intake considered a major factor in dysregulation of blood pressure in patients with hypertension and also in normotensive individuals who are at risk of developing cardiovascular disease [51,52]. In a meta-analysis involving 2600 normotensive and hypertensive subjects, a median dose of 75 mmol potassium/day (supplemented through diet) decreased systolic blood pressure by 3.11 mmHg and diastolic blood pressure by 1.97 mmHg [52,53]. The main source of potassium in humans is the diet, especially fresh fruits, vegetables, and milk, accounting for 40–100 mmol daily intake [54]. In 2012, WHO strongly recommended an increase in potassium intake from food to reduce blood pressure and risk of cardiovascular disease, stroke, and coronary heart disease and conditionally recommended a daily potassium intake of at least 90 mmol (3510 mg) for adults [55]. Although it is possible to achieve this target by consuming conventional foods such as rice and vegetables, these foods often have a high energy content or may not be consumable in sufficient amounts to confer the benefit. For example, a daily serving of approximately 300 g of brown rice or 700 g of spinach, two of the most potassium-rich foods, would provide the entire potassium requirements. *Kappaphycus*, with 20% potassium of its dry weight, would meet the same requirement with only ~18 g dried material. Therefore, the use of *Kappaphycus* as a functional food is a feasible intervention due to its high potassium content for individuals who are at risk of cardiovascular disease.

This study has demonstrated positive effects of *Kappaphycus* on gut microbiota, selecting against Firmicutes and promoting Bacteroidetes. In humans and animal models, obesity is associated with increases in the Firmicutes and decline in the Bacteroidetes [56,57]. The positive shift in gut microbiota associated with health benefits of certain diets has been attributed partly to dietary fiber [58,59]. Fiber may increase digesta mass, thereby shortening transit time and increasing defecation frequency as well as through increased fermentation leading to enhanced bacterial proliferation [60]. The increase in *Bacteroides* sp. and S24-7 family species following *Kappaphycus* supplementation may therefore be due to carrageenan. A recent study has shown that *Bacteroides* sp. are able to digest carrageenan [61], producing oligosaccharides which possess lipid-lowering properties [62]. Further, an in vitro study has confirmed the role of carrageenan from *Kappaphycus* as a prebiotic [63], which would give a plausible explanation for the improvements in gut microbiota.

Bacteroides thetaiotaomi liberated carbohydrate-degrading enzymes in the gastrointestinal environment which support the growth of other beneficial bacteria such as *Bifidobacterium longum* [64]. Strains of the S24-7 family, also known as *Candidatus* Homeothermaceae, produced short-chain fatty acids including acetate, propionate, and succinate [65], which confer benefits against metabolic syndrome. Further, many species from gut bacteria also produce vitamins in the B family including thiamine, riboflavin, niacin, pantothenate, biotin, and folate [66]. In a batch culture system inoculated with human feces and designed to mimic the distal colon, *Kappaphycus* stimulated the proliferation of bacteria producing short-chain fatty acids, suggesting that the increase in the S24-7 family in this study may be a mechanism for increasing production of short-chain fatty acids [63]. The decrease in *Clostridiaceae* is noteworthy since members of the *Clostridiaceae* family are associated with poor metabolic outcomes [67,68] and strategic targeting of the family is a feasible intervention for diet-induced obesity [69]. Paradoxically, the *Oscillospira* genus which has been positively associated with leanness or lower body mass index in humans [70] was more abundant in obese rats than the *Kappaphycus*-treated rats despite the decrease in body weight in the latter group. This discrepancy may be caused by differences between species as one study with high-fat diet-fed rats suggested that *Oscillospira* in the ileum predisposes the individual to obesity and metabolic disorders [71].

This study treated rats with 5% *Kappaphycus* in food as in our previous studies with other microalgae and seaweeds [8,33]. This dose of seaweed translates to ~30 g daily intake of red seaweed

for an adult human [72]; the average daily intake of seaweeds in Japanese people was 14.3 g [73]. The dried red seaweed contains 34.6% of dietary fiber equating to ~10 g of dietary fiber daily in humans. Mean dietary fiber intake of the US population for 1999–2008 was 13.1–16.1 g/day [74]. Further, the American Dietetic Association recommended a daily dietary fiber intake of 25g for adult females and 38 g for adult men [75]. The dietary fiber intake from red seaweed in this study falls within the recommended daily fiber intake when added to mean US intake, also providing more than the recommended potassium intake. Future studies with such seaweeds warrant measurement of short-chain fatty acids that are produced through the supply of dietary fiber. Moreover, measurement of intestinal permeability and plasma concentrations of endotoxins would provide more insight into the role of seaweeds in improving gut structure and function to improve metabolic syndrome.

5. Conclusions

This study demonstrated the potential of *Kappaphycus* as a functional food with possible application in the prevention of metabolic syndrome. We have further demonstrated that *Kappaphycus* may reverse metabolic syndrome through selective inhibition of obesogenic gut bacteria and promotion of health-promoting gut bacteria. There were no negative effects of *Kappaphycus* on any of the measured variables.

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Kappaphycus alvarezii as a Food Supplement Prevents Diet-Induced Metabolic Syndrome in Rats

Stephen Wanyonyi [1], Ryan du Preez [1,2], Lindsay Brown [1,2], Nicholas A. Paul [3] and Sunil K. Panchal [1,*]

[1] Functional Foods Research Group, Institute for Agriculture and the Environment, University of Southern Queensland, Toowoomba, QLD 4350, Australia; Stephen.Wanyonyi@usq.edu.au (S.W.); Ryan.duPreez@usq.edu.au (R.d.P.); Lindsay.Brown@usq.edu.au (L.B.)

[2] School of Health and Wellbeing, University of Southern Queensland, Toowoomba, QLD 4350, Australia

[3] Faculty of Science, Health, Education and Engineering, University of the Sunshine Coast, Maroochydore, QLD 4558, Australia; npaul@usc.edu.au

* Correspondence: Sunil.Panchal@usq.edu.au; Tel.: +61-7-4631-1012

Supplementary Materials:

Supplementary Table S1. *Kappaphycus* amino acid composition

Biomass properties (% *dry weight*)	Mean	SD
Histidine	0.09	0.01
Serine	0.80	0.01
Arginine	0.83	0.01
Glycine	0.80	0.02
Aspartic acid	1.71	0.03
Glutamic acid	1.80	0.03
Threonine	0.84	0.01
Alanine	0.92	0.01
Proline	0.70	0.01
Lysine	0.61	0.01
Tyrosine	0.20	0.01
Methionine	0.21	0.00
Valine	1.06	0.02
Isoleucine	0.79	0.01
Leucine	1.25	0.03
Phenylalanine	0.82	0.01

Values are mean ± SD, *n* = 2.

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Supplementary Table S2. *Kappaphycus* CHONPS and halogens

Biomass properties (% d.w.)	Mean	SD
Carbon (C)	14.85	4.96
Hydrogen (H)	2.58	0.91
Oxygen (O)	20.00	4.01
Nitrogen (N)	0.31	0.00
Phosphorus (P)	0.03	0.01
Sulfur (S)	3.11	1.01
Chlorine (Cl)	23.43	7.39
Bromine (Br)	0.12	0.01
Fluorine (F)	0.00	0.00
Iodine (I)	< 0.02	-

Values are mean ± SD, *n* = 2.

Supplementary Table S3. *Kappaphycus* metals and metalloids

Biomass properties (*mg/kg*)	Mean	SD
Aluminum (Al)	108.00	12.73
Arsenic (As)	4.27	0.11
Boron (B)	118.00	5.66
Barium (Ba)	1.27	0.29
Calcium (Ca)	8,964.00	599.63
Cadmium (Cd)	0.07	0.01
Cobalt (Co)	0.64	0.01
Chromium (Cr)	25.30	8.34
Copper (Cu)	1.90	0.97
Iron (Fe)	424.50	7.78
Mercury (Hg)	0.22	0.08
Potassium (K)	200,003.50	3,457.05
Magnessium (Mg)	5,692.50	208.60
Manganese (Mn)	4.67	0.73
Molybdenum (Mo)	<0.10	-
Sodium (Na)	37,692.50	239.71
Nickel (Ni)	7.84	4.19
Lead (Pb)	0.15	0.03
Selenium (Se)	<1.00	-
Strontium (Sr)	147.50	19.09
Vanadium (V)	4.13	0.26
Zinc (Zn)	4.46	-

Values are mean ± SD, *n* = 2.

CHAPTER 3. ι-CARRAGEENAN IN DIET-INDUCED METABOLIC SYNDROME

Sulphated polysaccharides including the carrageenans are common in seaweeds and are widely used in the food industry. The previous chapter showed that κ-carrageenan prevented symptoms of metabolic syndrome in rats. This chapter investigated the closely-related ι-carrageenan from the red seaweed, *Sarconema filiforme*, a linear polysaccharide with two sulphates per disaccharide unit. In contrast to the previous chapter, the potential of *S. filiforme* was evaluated in a reversal model of diet-induced metabolic syndrome in rats. There have been no studies investigating *S. filiforme* in metabolic syndrome. Therefore, the aim of this chapter was to determine the effectiveness of ι-carrageenan as a functional food to reverse metabolic syndrome. The current study is important given the high incidence and increasing prevalence of metabolic syndrome in humans around the world, highlighting an urgent need to treat affected individuals who have an increased risk of developing cardiovascular disease and type 2 diabetes. If the aim of this study is achieved, there is increased justification for the aquaculture of *S. filiforme* to supply a potential food market in addition to its industrial uses. This would be an advantage to regional Australia and neighbouring countries as this species of seaweed is widely distributed on the western and southern coasts of Australia and throughout the Indo-Pacific tropics and subtropics.

Carrageenans from the Red Seaweed *Sarconema filiforme* Attenuate Symptoms of Diet-Induced Metabolic Syndrome in Rats

Ryan du Preez [1,2,†] , **Nicholas Paul** [3] , **Peter Mouatt** [4] , **Marwan E. Majzoub** [5,6] , **Torsten Thomas** [5,6] , **Sunil K. Panchal** [1,‡] **and Lindsay Brown** [1,2,*]

1   Functional Foods Research Group, University of Southern Queensland, Toowoomba, QLD 4350, Australia; r.dupreez@cqu.edu.au (R.d.P.); S.Panchal@westernsydney.edu.au (S.K.P.)
2   School of Health and Wellbeing, University of Southern Queensland, Toowoomba, QLD 4350, Australia
3   School of Science and Engineering, University of the Sunshine Coast, Maroochydore, QLD 4558, Australia; npaul@usc.edu.au
4   Southern Cross Plant Science, Southern Cross University, Lismore, NSW 2480, Australia; Peter.Mouatt@scu.edu.au
5   Centre for Marine Science and Innovation, University of New South Wales, Sydney, NSW 2052, Australia; m.majzoub@unsw.edu.au (M.E.M.); t.thomas@unsw.edu.au (T.T.)
6   School of Biological, Earth and Environmental Sciences, University of New South Wales, Sydney, NSW 2052, Australia
*   Correspondence: Lindsay.Brown@usq.edu.au; Tel.: +61-7-4631-1319
†   Current address: School of Health, Medical and Applied Sciences, Central Queensland University, Rockhampton, QLD 4701, Australia.
‡   Current address: School of Science, Western Sydney University, Richmond, NSW 2753, Australia.

Abstract: Carrageenans are thickening and gelling agents that may provide health benefits. Iota (ι)-carrageenan, a linear sulfated polysaccharide, is produced by the red seaweed, *Sarconema filiforme*. This study investigated the potential of this seaweed as a functional food for the reversal of metabolic syndrome and possible mechanisms. Male Wistar rats were divided into four groups in a 16-week protocol: corn starch diet-fed rats (C); C rats supplemented with 5% *S. filiforme* for the last 8 weeks (CSF); high-carbohydrate, high-fat diet-fed rats (H); and H rats supplemented with 5% *S. filiforme* for the last 8 weeks (HSF). *S. filiforme* was produced in tank-based aquaculture yielding 27 g dry weight/day/m^2 of culture area. H rats developed obesity, hypertension, dyslipidaemia, glucose intolerance, fatty liver and increased left ventricular collagen deposition. *S. filiforme* supplementation decreased body weight, abdominal and liver fat, systolic blood pressure, plasma total cholesterol concentrations, and plasma activities of alanine transaminase and aspartate transaminase. *S. filiforme* supplementation modulated gut microbiota without changing the Firmicutes to Bacteroidetes ratio. *S. filiforme* improved symptoms of high-carbohydrate, high-fat diet-induced metabolic syndrome in rats. Possible mechanisms include a reduced infiltration of inflammatory cells into organs as well as prebiotic actions in the gastrointestinal tract.

Keywords: algae; *Sarconema filiforme*; sulfated polysaccharides; ι-carrageenan; prebiotics; gut microbiota; aquaculture; nutraceutical

1. Introduction

Carrageenans are a group of high molecular weight (>100 kDa) sulfated polygalactans isolated from red seaweeds (Rhodophyceae) that are "generally regarded as safe" for routine use as gelling and

thickening agents in foods [1]. The three types of carrageenans, named kappa (κ), iota (ι) and lambda (λ), have one, two and three sulfate groups per disaccharide unit, respectively [1]. The major commercially cultivated warm-water species for carrageenans are *Kappaphycus alvarezii* and *Eucheuma denticulatum* [2], producing κ- and ι-carrageenans, respectively. These seaweeds are grown on a commercial scale primarily in Indonesia, the Philippines, Malaysia, Brazil and Tanzania [3]. *Sarconema filiforme* is a red seaweed containing ι-carrageenan, distributed throughout the tropical and subtropical Indo-Pacific, including along the eastern and western coasts of Australia [4,5].

The health benefits of red seaweeds include obesity reduction, decreased lipid absorption and modification of the binding of cholesterol in the gastrointestinal tract thereby reducing cardiovascular disease risk [1,6]. However, there have been no studies on *Sarconema* species or their major constituents in metabolic syndrome for reduction of obesity, hypertension, fatty liver, hyperlipidaemia or diabetes. Recently, 0.12% ι-carrageenan has been used in a nasal spray in patients against rhinitis, demonstrating safety and efficacy [7]. In our previous study, rats fed with κ-carrageenan-producing *K. alvarezii* showed normalised body weight and adiposity, lowered systolic blood pressure, improved heart and liver structure, and lowered plasma lipids, compared to diet-induced obese rats [8].

The aim of the present study was to determine changes in parameters defining cardiovascular and metabolic health following chronic consumption of *S. filiforme*, produced through intensive tank-based cultivation, as a potential commercial source of ι-carrageenan. Firstly, we evaluated the proximate and elemental composition of *S. filiforme*. Secondly, a validated diet-induced rat model of metabolic syndrome that closely mimics the symptoms of human metabolic syndrome was used to measure cardiovascular and metabolic parameters. We measured systolic blood pressure, diastolic stiffness, cardiac inflammatory cells and collagen deposition in the heart for cardiovascular effects; plasma liver enzyme activities, inflammatory cells and fat vacuoles in the liver for liver effects; and body weight, total cholesterol and triglyceride concentrations, and glucose and insulin tolerance tests for metabolic effects. Further, we characterised the microbial composition of faecal samples after seaweed treatment, since functional foods may reverse obesity-induced cardiometabolic changes through alterations in the gut microbiota [9]. We hypothesised that 5% *S. filiforme* supplementation for the last 8 weeks will reverse the changes induced by the high-carbohydrate, high-fat diet to a greater extent than *K. alvarezii* due to the additional sulfate group in the disaccharide units of ι-carrageenan. The mechanisms of these effects could include the actions of ι-carrageenan as a prebiotic in the colon and to prevent infiltration of inflammatory cells into organs such as the heart and liver.

2. Results

2.1. Preparation of S. filiforme *Powder and Analysis*

S. filiforme samples were taken from batches grown in the Austral summer during January, February and March 2018 with an overall average yield of 27 g dry weight/day/m^2 (Figure 1A). This equates to weekly production of 189 g dry weight/m^2 of available culture area for intensive land-based production of the seaweed. In the current study, high-carbohydrate, high-fat diet-fed rats treated with *S. filiforme* (HSF) consumed 5% *S. filiforme* for the last eight weeks (~1.05 g/day). Using the Reagan-Shaw calculation for rat-to-human scaling, this equates to approximately 6.3 g dry weight of seaweed/day for humans [10]. For perspective, based on the overall average yield and with 42 m^2 of culture area, the University of the Sunshine Coast facility at Bribie Island could provide continuous seaweed at this dose for 179 people; at this overall yield, 5.7 km^2 of culture area could provide continuous seaweed for the current Australian population. The seaweed powder contained (in % dry weight): 34.4% carbohydrates (comprising 21.7% total dietary fibre, including 9.6% insoluble fibre and 12.2% of soluble fibre, almost all as ι-carrageenan), 11.8% protein, 1.4% lipid, 50.8% ash and 1.6% moisture (Table 1). Elemental analysis showed 21.2% C, 3.3% H, 2.6% N, 4.2% S, 18.9% K and 2.7% Na (Table 1). The energy content was 8.7 kJ/g.
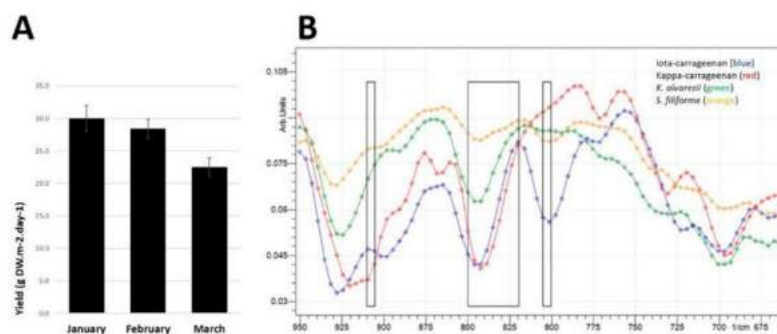
Figure 1. (**A**) Biomass yields of *Sarconema filiforme* between January and March 2018. Data show means ± SEM, n = 20–27 weekly growth measurements from 1000 L outdoor tanks from each month. (**B**) Attenuated Total Reflectance-Fourier-Transform Infrared Spectroscopy (ATR-FTIR) transmittance from 950 to 675 cm^{-1} of ι-carrageenan (blue line), κ-carrageenan (red line), *Kappaphycus alvarezii* (green line) and *Sarconema filiforme* (orange line). Far left rectangle showing 900–905 cm^{-1}, middle rectangle showing 820-850 cm^{-1} and far right rectange showing 800–805 cm^{-1}.

Table 1. Biochemical composition of *Sarconema filiforme* biomass.

Proximate	% dry weight	Metals	mg/kg
Carbohydrate	34.4		
Protein	11.8	Aluminium	39.3
Lipid	1.4	Antimony	0.1
Ash	50.8	Arsenic	6.2
Moisture	1.6	Barium	0.5
Energy (kJ/g)	8.7	Boron	299
Fibre	**% dry weight**	Cadmium	0.1
Total dietary fibre	21.7	Calcium	2150
Insoluble dietary fibre	9.6	Chromium	0.3
Soluble dietary fibre (by difference)	12.2	Cobalt	0.6
Ultimate	**% dry weight**	Copper	1.6
Carbon	21.2	Iron	1875
Nitrogen	2.6	Lead	0.2
Hydrogen	3.3	Magnesium	3655
Sulfur	4.2	Manganese	20.3
Amino acids	**% dry weight**	Mercury	0.3
Total amino acids	11.8	Molybdenum	0.2
Histidine	0.2	Nickel	0.7
Serine	0.7	Phosphorus	2500
Arginine	1.0	Potassium	189,500
Glycine	0.7	Selenium	0.3
Aspartic acid	1.3	Silver	0.0
Glutamic acid	1.8	Sodium	27,000
Threonine	0.6	Strontium	31.3
Alanine	0.8	Tin	0.1
Proline	0.6	Vanadium	2.4
Lysine	0.6	Zinc	128
Tyrosine	0.3	**Fatty acids**	**% dry weight**
Methionine	0.2		
Valine	0.8	Total fatty acids	1.1
Isoleucine	0.7	C16:0 palmitic	0.4
Leucine	1.0	C20:4 ω-6 arachidonic	0.5
Phenylalanine	0.6	C20:5 ω-3 eicosapentaenoic	0.1

Values are mean of two sub-samples of blended biomass.

2.2. *ATR-FTIR of* S. filiforme *and* K. alvarezii

The normalised transmission spectra of reference samples of ι-carrageenan and κ-carrageenan are given with those of *S. filiforme* and *K. alvarezii* (Figure 1B). The peak at approximately 800–805 cm^{-1} is most prominent in ι-carrageenan, which is indicative of 3,6-anhydro-ᴅ-galactose 2-sulfate and the bands between 820 and 850 cm^{-1} are indicative of galactose 2-sulfate, galactose 4-sulfate and galactose 2,6-disulfate, which are characteristic of carrageenans. The band at ~800–805 cm^{-1} is more prominent in ι-carrageenan and *S. filiforme* samples. The band at ~905–910 cm^{-1} is indicative of anhydro-galactose-2-sulfate which is consistent between ι-carrageenan and *S. filiforme* samples.

2.3. *Physiological and Metabolic Responses*

Food intake was higher in corn starch diet-fed rats (C) compared to high-carbohydrate, high-fat diet-fed rats (H). HSF had lower food intake than H rats (Table 3). The body weight of H rats was greater than C rats and that of HSF rats was lower than H rats (Figure 2A). Lean mass was unchanged in all groups. Fat mass measurements were consistent with body weight measurements (Table 3).
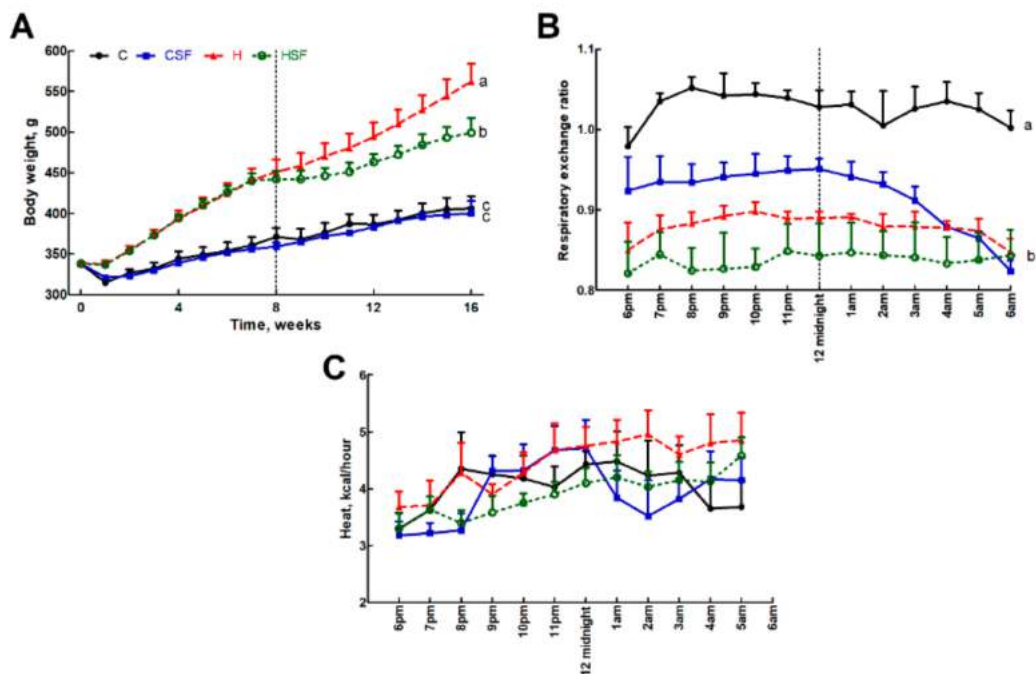
Figure 2. (**A**) Body weight, (**B**) 12-hour indirect calorimeter data for respiratory exchange ratio and (**C**) heat production in corn starch diet-fed rats (C), corn starch diet-fed rats supplemented with *Sarconema filiforme* (CSF), high-carbohydrate, high-fat diet-fed rats (H) and high-carbohydrate, high-fat diet-fed rats supplemented with *Sarconema filiforme* (HSF). End-point means with unlike superscripts differ (a, b or c), *p* < 0.05.

Table 2. Responses to *Sarconema filiforme*.

Variables	C	CSF	H	HSF	Diet	*p* value Treatment	Interaction
Physiological variables							
0 week body weight, *g*	338 ± 1	338 ± 1	339 ± 1	338 ± 1	0.66	0.66	0.66
8 week body weight, *g*	371 ± 11[b]	359 ± 5[b]	451 ± 15[a]	442 ± 10[a]	<0.0001	0.31	0.88
16 week body weight, *g*	405 ± 4[c]	398 ± 5[c]	550 ± 15[a]	498 ± 10[b]	<0.0001	0.004	0.025
16 week lean mass, *g*	321 ± 9	324 ± 7	309 ± 10	338 ± 12	0.92	0.13	0.22
16 week fat mass, *g*	59 ± 4[c]	58 ± 6[c]	251 ± 30[a]	151 ± 16[b]	<0.0001	0.004	0.005
8 week lean/fat mass proportion	4.4 ± 0.5[b]	6.8 ± 0.8[a]	2.2 ± 0.4[c]	2.3 ±0.2[c]	<0.0001	0.056	0.08
16 week lean/fat mass proportion	5.5 ± 0.4[b]	6.0 ± 0.6[a]	1.3 ± 0.1[d]	2.5 ± 0.4[c]	<0.0001	0.047	0.93
16 week bone mineral content, *g*	12.3 ± 0.8[c]	12.1 ± 0.5[c]	17.6 ± 0.7[a]	14.6 ± 0.6[b]	<0.0001	0.021	0.041
16 week bone mineral density, *g/cm²*	0.180 ± 0.002	0.183 ± 0.004	0.184 ± 0.005	0.184 ± 0.002	0.53	0.70	0.70
Food intake 0-8 weeks, *g/day*	41.4 ± 0.9[a]	39.0 ± 1.4[a]	27.8 ± 0.9[b]	26.5 ± 1.3[b]	<0.0001	0.21	0.71
Food intake 9-16 weeks, *g/day*	37.1 ± 0.4[a]	37.0 ± 1.1[a]	26.2 ± 0.7[b]	20.9 ± 0.8[c]	<0.0001	0.012	0.015
Water intake 0-8 weeks, *g/day*	40.8 ± 4.4[a]	32.7 ± 2.8[b]	25.5 ± 0.8[c]	29.3 ± 2.7[b]	0.0061	0.51	0.07
Water intake 9-16 weeks, *g/day*	30.4 ± 3.8[b]	42.3 ± 1.4[a]	22.2 ± 0.6[c]	41.8 ± 2.1[a]	0.07	<0.0001	0.11
Energy intake 0-8 weeks, *kJ/day*	468 ± 9[b]	438 ±16[b]	593 ± 13[a]	597 ± 35[a]	<0.0001	0.65	0.55
Energy intake 9-16 weeks, *kJ/day*	415 ± 4[b]	407 ± 13[b]	558 ± 10[a]	533 ± 17[a]	<0.0001	0.18	0.93
16 week abdominal circumference, *cm*	20.1 ± 0.4[c]	19.4 ± 0.1[c]	23.8 ± 0.5[a]	21.2 ± 0.3[b]	<0.0001	<0.0001	0.005
Body mass index, *g/cm²*	0.61 ± 0.02[c]	0.65 ± 0.01[c]	0.81 ± 0.03[a]	0.74 ± 0.02[b]	<0.0001	0.46	0.011
Retroperitoneal fat, *mg/mm*	216 ± 37[c]	196 ± 15[c]	636 ± 67[a]	423 ± 41[b]	<0.0001	0.007	0.025
Epididymal fat, *mg/mm*	78 ± 18[c]	62 ± 9[c]	191 ± 28[a]	116 ± 12[b]	<0.0001	0.007	0.07
Omental fat, *mg/mm*	147 ± 21[c]	142 ± 12[c]	333 ± 29[a]	245 ± 16[b]	<0.0001	0.019	0.035
Visceral adiposity, %	5.1 ± 0.6[c]	4.8 ± 0.4[c]	10.0 ± 0.6[a]	7.8 ± 0.3[b]	<0.0001	0.011	0.048
Liver wet weight, *mg/mm*	218 ± 7[b]	231 ± 7[b]	365 ± 26[a]	343 ± 16[a]	<0.0001	0.77	0.27
Cardiovascular variables							
8 week systolic blood pressure, *mmHg*	123 ± 4[b]	122 ± 3[b]	135 ± 3[a]	138 ± 2[a]	0.0001	0.76	0.54
16 week systolic blood pressure, *mmHg*	128 ± 3[b]	126 ± 4[b]	145 ± 4[a]	132 ± 3[b]	0.007	0.07	0.18
Left ventricle + septum wet weight, *mg/mm*	22.6 ± 2.1	22.5 ± 1.0	21.2 ± 2.2	21.5 ± 0.6	0.38	0.94	0.88
Right ventricle, *mg/mm*	4.1 ± 0.3	3.5 ± 0.3	4.4 ± 0.1	4.3 ± 0.2	0.06	0.22	0.38
Left ventricular diastolic stiffness (κ)	21.2 ± 2.3[c]	21.7 ± 1.7[c]	29.6 ± 1.4[a]	25.6 ± 1.9[b]	0.022	0.36	0.24
Left ventricle collagen area, %	8.1 ± 2.4[c]	8.3 ± 1.8[c]	18.4 ± 1.9[a]	12.1 ± 1.7[b]	0.009	0.11	0.11
Left ventricle inflammatory cells, *cells/200μm²*	7 ± 2[c]	9 ± 2[c]	25 ± 3[a]	16 ± 3[b]	0.0004	0.1949	0.0520

Table 3. Responses to *Sarconema filiforme*.

Variables	C	CSF	H	HSF	Diet	*p* Value Treatment	Interaction
Metabolic variables							
Plasma total cholesterol, *mmol/L*	1.59 ± 0.06[b]	1.44 ± 0.06[b]	1.73 ± 0.09[a]	1.51 ± 0.08[b]	0.18	0.021	0.65
Plasma triglycerides, *mmol/L*	0.50 ± 0.05[b]	0.52 ± 0.08[b]	1.15 ± 0.13[a]	1.04 ± 0.21[a]	<0.0001	0.73	0.62
Plasma non-esterified fatty acids, *mmol/L*	0.68 ± 0.12[b]	0.74 ± 0.24[b]	2.71 ± 0.29[a]	1.99 ± 0.59[a]	<0.0001	0.33	0.25
Alanine transaminase, *U/L*	39 ± 5[ab]	33 ± 5[ab]	50 ± 11[ab]	31 ± 2[b]	0.55	0.010	0.39
Aspartate transaminase, *U/L*	138 ± 20[ab]	125 ± 20[b]	174 ± 17[a]	128 ± 13[b]	0.29	0.011	0.37
Liver inflammatory cells, *cells/200μm²*	11 ± 2	13 ± 2	30 ± 3[a]	16 ± 3[b]	0.0003	0.029	0.005
Liver fat vacuoles area, *μm²*	12.4 ± 1.2	10.7 ± 1.8	90.6 ± 7.1[a]	70.4 ± 2.7[b]	<0.0001	0.012	0.029
Oral glucose tolerance test							
0 week basal blood glucose, *mmol/L*	2.6 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	2.5 ± 0.1	0.66	0.66	0.66
0 week area under the curve, *mmol/L×min*	751 ± 26	650 ± 23	727 ± 23	689 ± 18	0.76	0.007	0.20
8 week basal blood glucose, *mmol/L*	2.5 ± 0.1[b]	2.8 ± 0.1[b]	3.9 ± 0.4[a]	3.4 ± 0.1[a]	<0.0001	0.60	0.044
8 week 120-min blood glucose, *mmol/L*	3.6 ± 0.4[b]	3.6 ± 0.1[b]	5.2 ± 0.1[a]	5.1 ± 0.1[a]	<0.0001	0.78	0.78
8 week area under the curve, *mmol/L×min*	559 ± 34[b]	538 ± 18[b]	690 ± 17[a]	645 ± 12[a]	<0.0001	0.11	0.56
16 week basal blood glucose, *mmol/L*	2.6 ± 0.1[b]	2.6 ± 0.2[b]	3.5 ± 0.3[a]	3.3 ± 0.2[a]	0.002	0.67	0.67
16 week 120-min blood glucose, *mmol/L*	3.6 ± 0.1[b]	3.8 ± 0.2[b]	5.8 ± 0.5[a]	5.0 ± 0.1[a]	<0.0001	0.22	0.043
16 week area under the curve, *mmol/L×min*	506 ± 22[c]	547 ± 8[b]	675 ± 20[a]	647 ± 18[a]	<0.0001	0.71	0.056
Insulin tolerance test							
8 week 120-min blood glucose, *mmol/L*	2.1 ± 0.7[b]	2.5 ± 0.5[b]	3.8 ± 0.5[a]	4.2 ± 0.4[a]	0.004	0.47	1.00
8 week area under the curve, *mmol/L×min*	204 ± 33[b]	238 ± 36[b]	349 ± 23[a]	377 ± 24[a]	0.0002	0.37	0.93
16 week 120-min blood glucose, *mmol/L*	3.6 ± 0.4	3.3 ± 1.1	4.3 ± 1.1	3.4 ± 0.3	0.66	0.52	0.74
16 week area under the curve, *mmol/L×min*	271 ± 64	225 ± 36	396 ± 56	341 ± 19	0.006	0.23	0.91

Values are presented as mean ± SEM, n = 10–12. Means in a row with unlike superscripts differ (a, b or c), *p* < 0.05. C, corn starch diet-fed rats; CSF, corn starch diet-fed rats supplemented with *Sarconema filiforme*; H, high-carbohydrate, high-fat diet-fed rats; HSF, high-carbohydrate, high-fat diet-fed rats supplemented with *Sarconema filiforme*.

C rats had higher respiratory exchange ratio compared to H rats, while corn starch diet-fed rats treated with *S. filiforme* (CSF) and HSF were the same as H rats (Figure 2B). C rats had lower heat production than H rats, while CSF was higher than C and HSF was lower than H (Figure 2C). Total abdominal fat was highest in H rats followed by HSF, CSF and C rats.

After eight weeks, systolic blood pressures of H diet-fed groups (H and HSF) were higher than C diet-fed groups (C and CSF). Systolic blood pressures in H rats were higher at 16 weeks than in C rats. HSF rats had decreased systolic blood pressures compared to H control rats. Diastolic stiffness was higher in H rats compared to C rats. HSF rats showed normalised diastolic stiffness. Left ventricular with septum wet weights and right ventricular wet weights were unchanged across all groups. Left ventricles from H rats showed increased infiltration of inflammatory cells and collagen deposition, whereas these changes were not seen in left ventricles from C rats (Table 3). Infiltration of inflammatory cells and collagen deposition was normal in hearts from CSF rats and decreased in HSF rats (Figure 3 and Table 3).
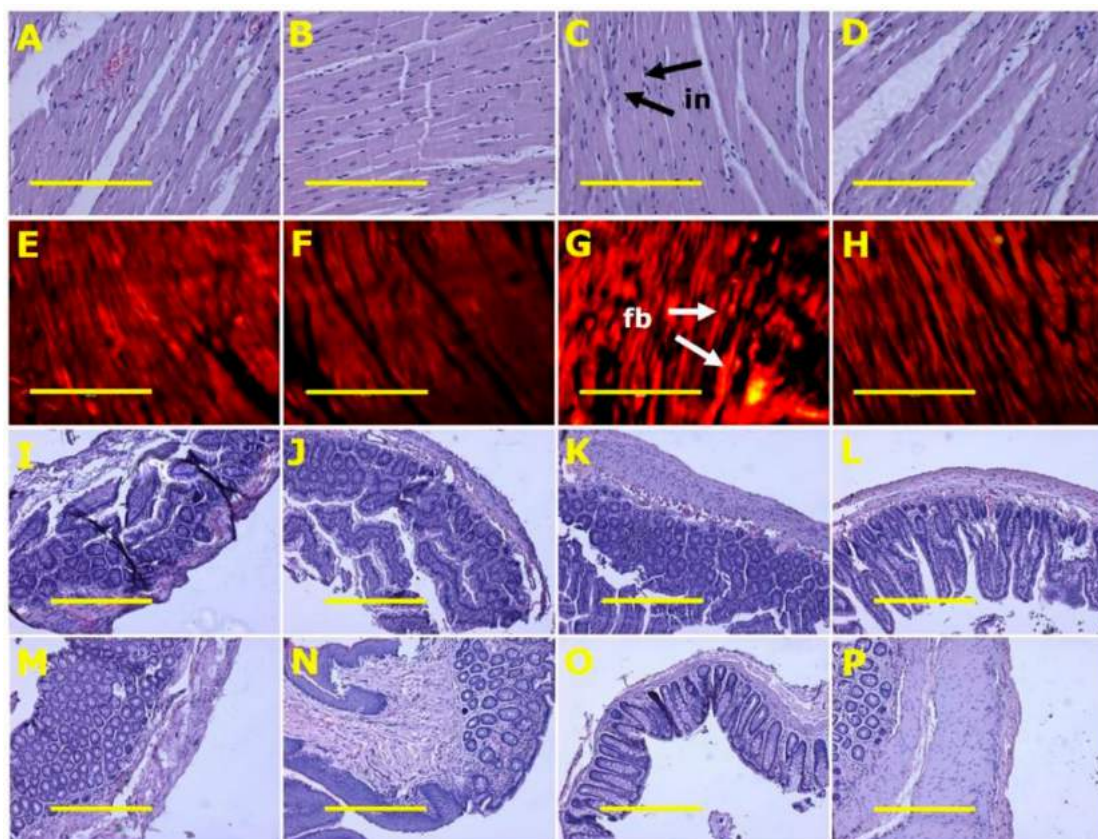
Figure 3. Heart inflammation (**A**–**D**) using haematoxylin and eosin stain; heart fibrosis (**E**–**H**) using picrosirius red stain; ileum (**I**–**L**) and colon (**M**–**P**) structure using haematoxylin and eosin stain in corn starch diet-fed rats (**A**,**E**,**I**,**M**), corn starch diet-fed rats supplemented with *Sarconema filiforme* (**B**,**F**,**J**,**N**), high-carbohydrate, high-fat diet-fed rats (**C**,**G**,**K**,**O**) and high-carbohydrate, high-fat diet-fed rats supplemented with *Sarconema filiforme* (**D**,**H**,**L**,**P**). Fibrosis = fb; inflammation = in. Scale bar for images A-H is 200μm (20×) and for images I-P is 100μm (10×).

Plasma triglyceride concentrations were higher in H and HSF rats compared to C and CSF rats; plasma total cholesterol concentrations were highest in H rats and lowest in C, CSF and HSF rats; and plasma non-esterified fatty acids were unchanged across all groups (Table 3). C rats had lower basal blood glucose concentrations compared to H rats. Intervention with *S. filiforme* reduced basal blood glucose concentrations. The blood glucose area under the curve was not different between groups (Table 3).

H rats had higher plasma activities of ALT and AST compared to C rats. CSF and HSF rats were the same as C rats (Table 3). Livers from H rats showed increased fat deposition and infiltration of inflammatory cells compared to livers from C rats (Table 3 and Figure 4). HSF rats had reduced fat deposition compared to H rats (Figure 4).
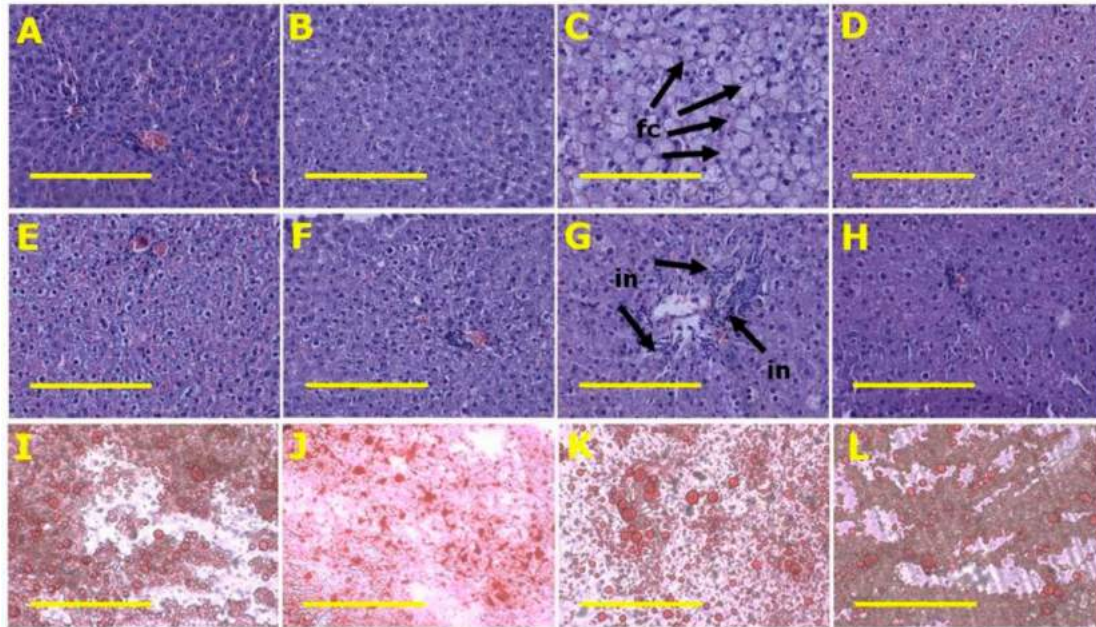
Figure 4. Fat vacuoles (**A–D**) and inflammation (**E–H**) using haematoxylin and eosin and liver fat using oil red O stain (**I–L**) in corn starch diet-fed rats (**A,E,I**), corn starch diet-fed rats supplemented with *Sarconema filiforme* (**B,F,J**), high-carbohydrate, high-fat diet-fed rats (**C,G,K**) and high-carbohydrate, high-fat diet-fed rats supplemented with *Sarconema filiforme* (**D,H,L**). Fat vacuole containing cells = fc; inflammatory cells = in. Scale bar is 200µm (20×).

2.4. Gut Structure and Microbiota

Histology of ileum and colon did not show any structural abnormalities in the experimental groups with normal crypt depth, villi length and goblet cells, and less inflammatory cell infiltration (Figure 3).

The gut microbiota was characterised by a total of 739,069 quality-filtered sequences that were clustered into 1233 zero-radius operational taxonomic units (zOTUs), which are roughly equivalent to the taxonomic level of species or strains. The calculated rarefaction curves based on rarefied and unrarefied data as well as Good's coverage of 99.67 ± 0.10% showed that the bacterial communities were almost fully recovered by the surveying effort.

There was no difference in Shannon's diversity or richness between the four groups (Figure 5). Diet and seaweed supplement both affected the overall bacterial community structure based on Bray-Curtis dissimilarity (Figure 6, Table 4; PERMANOVA, both $p = 0.0001$), and there was an interaction between the two factors (Figure 6, Table 4; PERMANOVA, $p = 0.003$). There were pairwise differences between the C and H groups indicating an effect of basal feed on the bacterial community structure ($p = 0.0017$). The addition of *S. filiforme* changed the bacterial communities (CSF, $p = 0.0014$; HSF, $p = 0.0396$). Bacterial communities in the CSF group were more variable compared to the C group (Figure 6, Table 4; PERMDISP; $p = 0.022$).

C rats and CSF rats had lower ratios of Firmicutes to Bacteroidetes (F/B ratio) compared to H and HSF rats (Figure 7). There was no effect of *S. filiforme* supplementation on the F/B ratio under either diet.
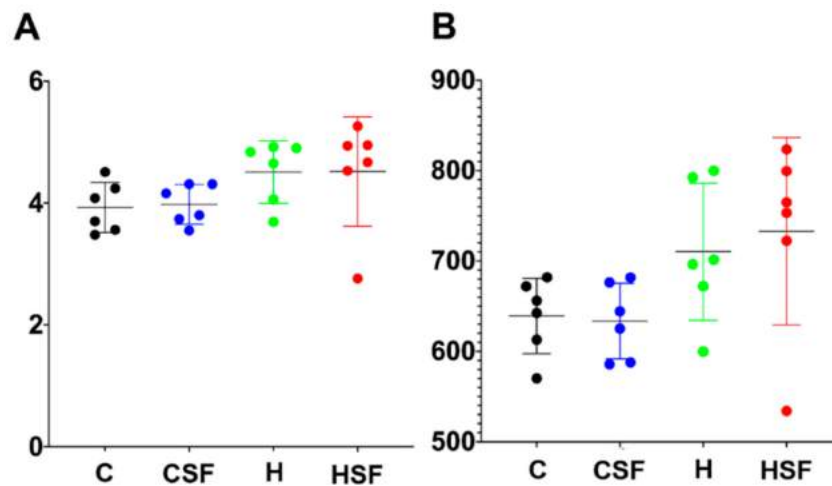
Figure 5. (**A**) Shannon diversity and (**B**) richness of faecal samples. C, corn starch diet-fed rats; CSF, corn starch diet-fed rats supplemented with *Sarconema filiforme*; H, high-carbohydrate, high-fat diet-fed rats; HSF, high-carbohydrate, high-fat diet-fed rats supplemented with *Sarconema filiforme*.
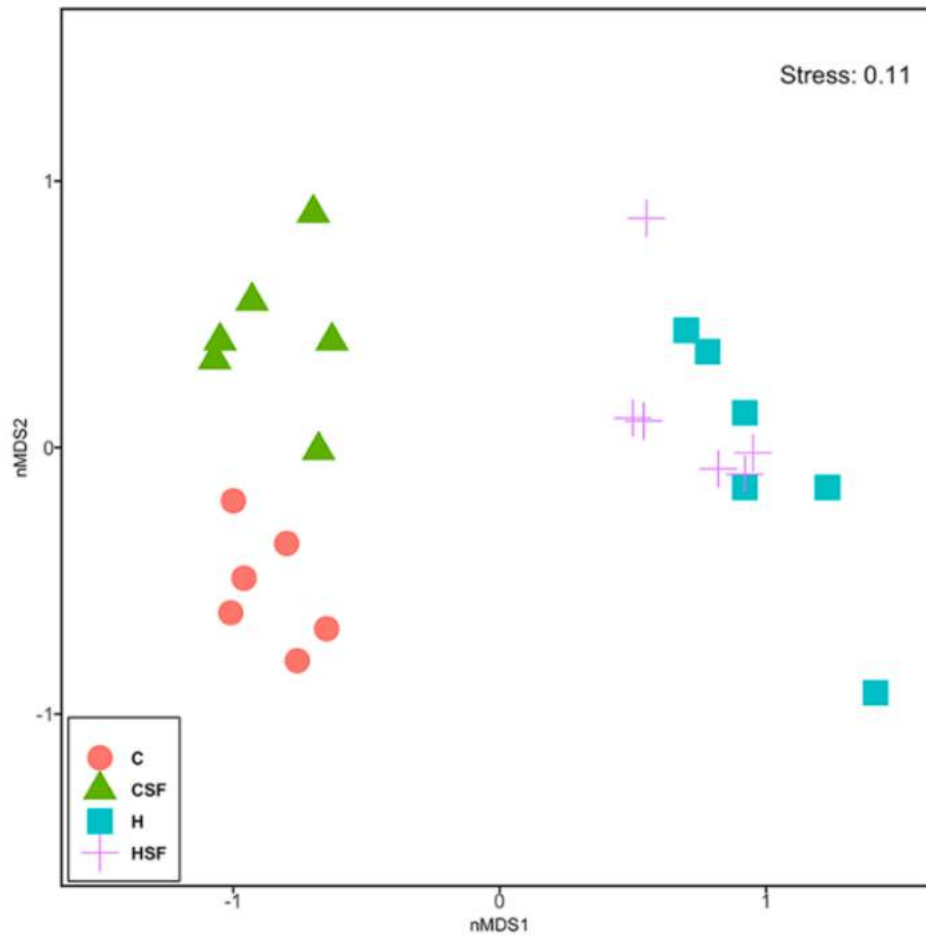
Figure 6. Multi-disciplinary scaling (MDS) plot of bacterial community structure of faecal samples from different feeding regimes. C, corn starch diet-fed rats; CSF, corn starch diet-fed rats supplemented with *Sarconema filiforme*; H, high-carbohydrate, high-fat diet-fed rats; HSF, high-carbohydrate, high-fat diet-fed rats supplemented with *Sarconema filiforme*.

Table 4. PERMANOVAs based on Bray-Curtis similarity measure for square-root transformed abundances of all rat faecal samples.

PERMANOVA						
Source	**df**	**SS**	**MS**	**Pseudo-F**	**P(perm)**	**Unique perms**
Diet	1	9306.2	9306.2	9.3671	0.0001	9912
Treatment	1	2477.6	2477.6	2.4938	0.0001	9860
Diet × treatment	1	1671.4	1671.4	1.6823	0.003	9836
Res	19	18876	993.5			
Total	22	32369				
PAIR-WISE TESTS						
Source			**t**		**P(perm)**	**Unique perms**
C, CSF			1.626		0.0014	462
C, H			2.4472		0.0017	462
C, HSF			2.544		0.0024	462
CSF, H			2.3327		0.0018	462
CSF, HSF			2.2125		0.0022	461
H, HSF			1.185		0.0396	462
PERMDISP (PAIRWISE COMPARISONS)						
Groups				**t**		**P(perm)**
C, CSF				1.7793		0.022
C, H				0.77115		0.65
C, HSF				0.80101		0.60
CSF, H				0.01072		0.99
CSF, HSF				1.6113		0.24
H, HSF				1.139		0.35

p values were calculated using 9,999 permutations under a residual model. C, corn starch diet-fed rats; CSF, corn starch diet-fed rats supplemented with *Sarconema filiforme*; H, high-carbohydrate, high-fat diet-fed rats; HSF, high-carbohydrate, high-fat diet-fed rats supplemented with *Sarconema filiforme*.

Figure 7. Effect of supplementation of diet (C or H) with *Sarconema filiforme* on the ratio of Firmicutes and Bacteroidetes abundances in rat faecal samples. Statistical analysis performed using ANOVA with Tukey's post hoc test for multiple comparisons. C, corn starch diet-fed rats; CSF, corn starch diet-fed rats supplemented with *Sarconema filiforme*; H, high-carbohydrate, high-fat diet-fed rats; HSF, high-carbohydrate, high-fat diet-fed rats supplemented with *Sarconema filiforme*.

2.5. Taxonomic Structure of the Bacterial Communities

The most abundant bacterial classes found in the faecal samples for different treatment groups were Bacteroidia, Bacilli, Clostridia, Erysipelotrichia and Verrucomicrobia (Figure 8). Other bacterial

classes, including Actinobacteria, Coriobacteriia, Melainabacteria, Deferribacteres, Saccharimonadia, Alphaproteobacteria, Deltaproteobacteria, Gammaproteobacteria and Mollicutes, were observed at lower abundance levels (<1%) in some (but not all) faecal samples.

Figure 8. Taxonomic profiles of bacterial communities shown at the class level of all faecal samples. C, corn starch diet-fed rats; CSF, corn starch diet-fed rats supplemented with *Sarconema filiforme*; H, high-carbohydrate, high-fat diet-fed rats; and HSF, high-carbohydrate, high-fat diet-fed rats supplemented with *Sarconema filiforme*.

The relative abundance of bacteria from the class Bacteroidia was reduced in H rats (15.03% to 17.07%) compared to C rats (29.98% to 32.68%) ($p < 0.001$). An increase in the relative abundance of bacteria from class Bacilli was observed in CSF, H and HSF rats (2.01% to 2.89%) compared to the C rats (0.58%; $p > 0.05$). A higher abundance of bacteria from the class Clostridia was observed for H rats (66.51% to 68.76%) compared to C rats (43.47% to 44.59%) ($p < 0.0001$) (Figure 8). An increase in the relative abundance of bacteria from the class Verrucomicrobiae and Erysipelotrichia was observed in C rats (Verrucomicrobiae: 10.74% to 13.92%; Erysipelotrichia: 6.17% to 9.30%) ($p > 0.05$) compared to H rats (Verrucomicrobiae: 8.83% to 9%; Erysipelotrichia: 3.86% to 4.33%) ($p > 0.05$; Figure 8).

Analysis of the bacterial community structure at the family level showed that Bacteroidaceae (class Bacteriodia), Muribaculaceae (class Bacteriodia), Prevotellaceae (class Bacteroidia), Lactobacillaceae (class Bacilli), Clostridiaceae 1 (class Clostridia), Lachnospiraceae (class Clostridia), Peptostreptococcaceae (class Clostridia), Ruminococcaceae (class Clostridia), Erysipelotrichaceae (class Erysipelotrichia) and Akkermansiacaeae (class Verrucomicrobia) were most dominant in the faecal samples (Figure 9). The relative abundance of bacteria from the family Ruminococcaceae was reduced for H rats (8.83% to 9%) compared to C rats (10.74% to 13.92%) ($p > 0.05$). A high abundance of bacteria from the family Lachnospiraceae was detected in H rats and HSF rats (36.37% to 39.43%, $p < 0.0001$) compared to C rats (15.17% to 15.26%). In contrast, the abundance of bacteria from the family Muribaculaceae was reduced in H rats (9.16% to 10.28%) compared to C rats (21.27% to 22.77%, $p < 0.05$) (Figure 9). Moreover, lower abundance of bacteria from the family Lactobacillaceae was observed for C rats (0.16%) compared to CSF, H and HSF (1.56% to 2.49%) ($p > 0.05$).

Analysis of the bacterial community structure at the genus level showed that *Bacteroides* (family Bacteroidaceae), unclassified Muribaculaeceae, *Clostridium sensu stricto 1* (family Clostridiaceae), *Lachnospiraceae* NK4A136 group (family Lachnospiraceae), *Roseburia* (family Lachnospiraceae), *Ruminococcus 1* (family Ruminococcaceae), unclassified Ruminococcaceae, *Turicibacter* (family Erysipelotrichaceae) and *Akkermansia* (family Akkermansiaceae) were most dominant in the faecal samples (Figure 10).
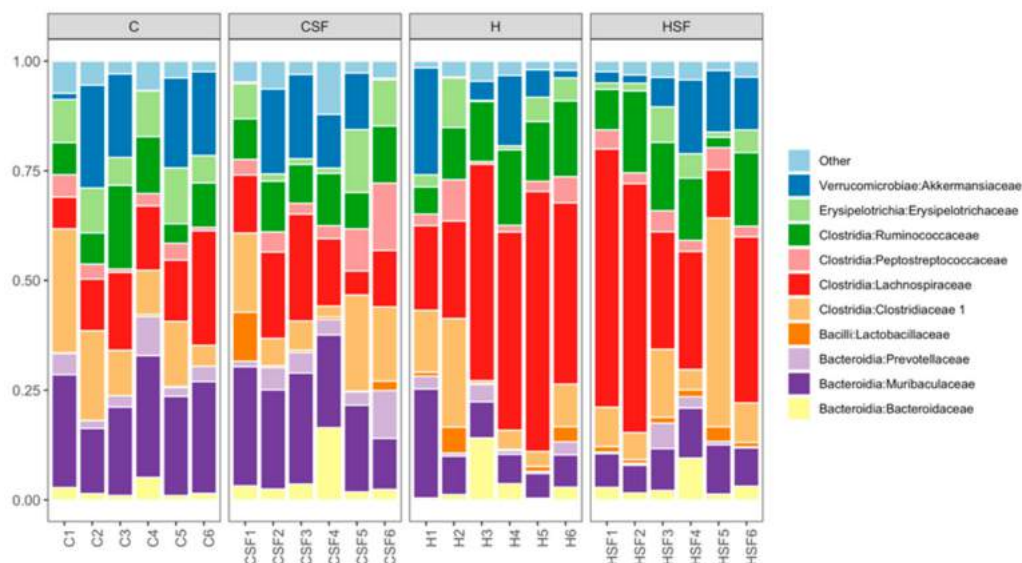
Figure 9. Taxonomic profiles of bacterial communities shown at the family level of all faecal samples. C, corn starch diet-fed rats; CSF, corn starch diet-fed rats supplemented with *Sarconema filiforme*; H, high-carbohydrate, high-fat diet-fed rats; HSF, high-carbohydrate, high-fat diet-fed rats supplemented with *Sarconema filiforme*.
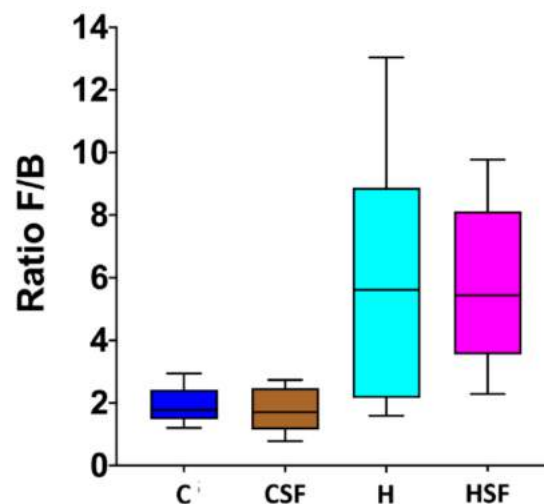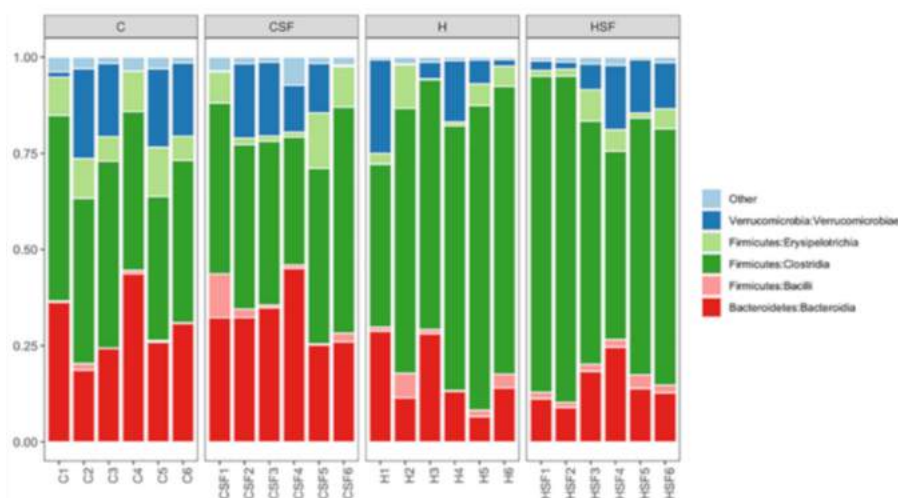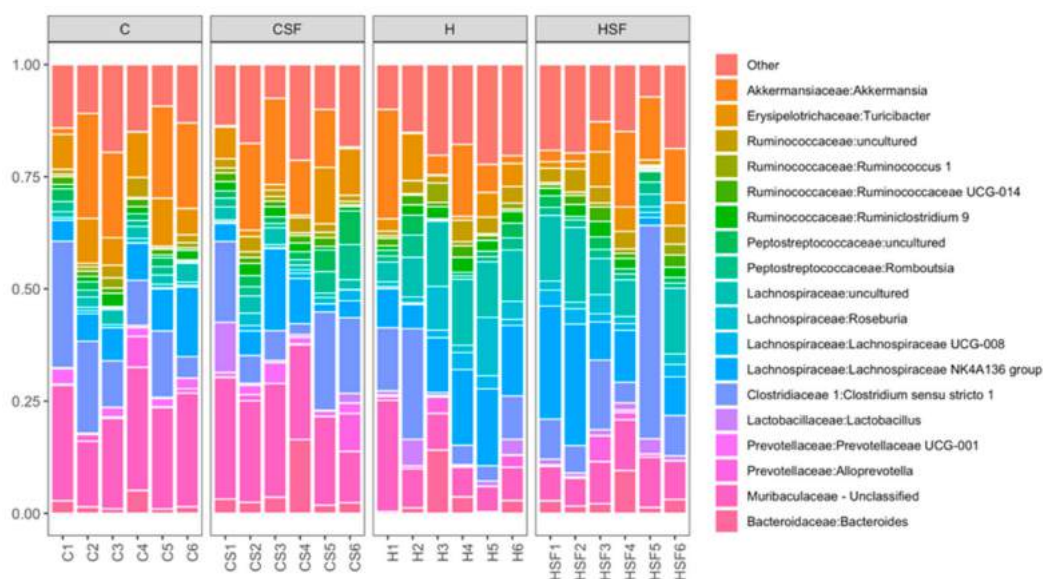

Figure 10. Taxonomic profiles of bacterial communities shown at the genus level of all faecal samples. C, corn starch diet-fed rats; CSF, corn starch diet-fed rats supplemented with *Sarconema filiforme*; H, high-carbohydrate, high-fat diet-fed rats; HSF, high-carbohydrate, high-fat diet-fed rats supplemented with *Sarconema filiforme*.

2.6. Multivariate Analysis of Physiological Data

A total of 23 physiological parameters were assessed and included in the analysis below (body weight, fat mass, lean mass, water intake, food intake, energy intake, feed efficiency, left ventricle with septum wet weight, right ventricle wet weight, retroperitoneal fat, omental fat, epididymal fat, total abdominal fat, liver wet weight, kidney wet weight, spleen wet weight, plasma non-esterified fatty acids, plasma triglycerides, systolic blood pressure, oral glucose tolerance area under the curve, oral glucose tolerance 120 min blood glucose concentrations, plasma aspartate transaminase activity and plasma alanine transaminase activity) for rats fed with the C and H diets and supplemented with *S. filiforme* (Table 5).

Table 5. PERMANOVAs based on Euclidean distance matrix for physiological data of all rat faecal samples.

PERMANOVA						
Source	**df**	**SS**	**MS**	**Pseudo-F**	**P(perm)**	**Unique perms**
Diet	1	4081700	4081700	53.366	0.0001	9924
Treatment	1	435050	435050	5.6882	0.0154	9942
Diet × treatment	1	408770	408770	5.3446	0.0187	9939
Res	20	1529700	76484			
Total	23	6455200				
PAIR-WISE TESTS						
Source				**t**	**P(perm)**	**Unique perms**
C, CSF				0.6848	0.82	462
C, H				5.6852	0.0018	461
C, HSF				4.4192	0.0024	462
CSF, H				5.8892	0.0021	462
CSF, HSF				4.7191	0.0023	462
H, HSF				2.5161	0.0268	462
PERMDISP (PAIRWISE COMPARISONS)						
Groups				**t**	**P(perm)**	
C, CSF				1.5351	0.23	
C, H				1.9906	0.11	
C, HSF				1.4159	0.20	
CSF, H				2.4448	0.0398	
CSF, HSF				2.2429	0.0139	
H, HSF				1.0507	0.29	

p values were calculated using 9999 permutations under a residual model. C, corn starch diet-fed rats; CSF, corn starch diet-fed rats supplemented with *Sarconema filiforme*; H, high-carbohydrate, high-fat diet-fed rats; HSF, high-carbohydrate, high-fat diet-fed rats supplemented with *Sarconema filiforme*.

Distance-based multivariate analysis showed that treatments have distinct responses on the physiological parameters. Diet and supplement affected the rats' physiological variables (Figure 11, Table 5; PERMANOVA; *p* = 0.0001 and *p* = 0.0154, respectively) and there was an interaction between the two factors (Figure 11, Table 5; PERMANOVA, *p* = 0.0187).
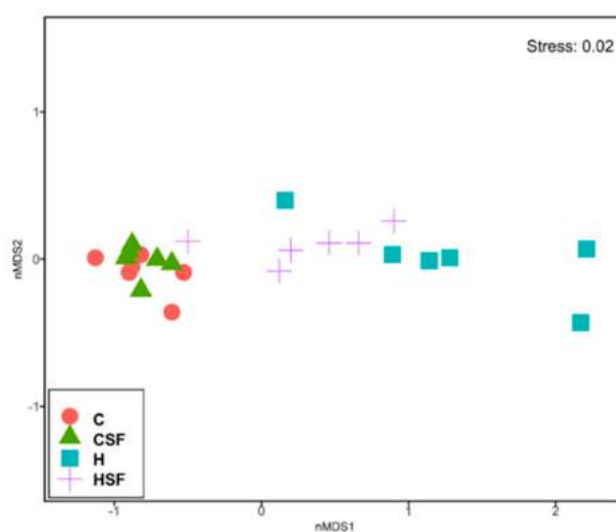
Figure 11. Non-metric multi-disciplinary scaling (nMDS) plot of physiological data from all physiological parameters measured after 16 weeks of feeding. C, corn starch diet-fed rats; CSF, corn starch diet-fed rats supplemented with *Sarconema filiforme*; H, high-carbohydrate, high-fat diet-fed rats; HSF, high-carbohydrate, high-fat diet-fed rats supplemented with *Sarconema filiforme*.

There was statistical support for differences between C and H rats without *S. filiforme* (p = 0.0018, Table 5) indicating an effect of basal diet on the overall physiological variables. There was also an effect for the addition of *S. filiforme* to the H diet (p = 0.0268; Figure 11, Table 5), however supplementation had no effect for C diet. Rat physiological variables in H and HSF rats were more variable between replicates compared to the CSF rats (Figure 11, Table 5; PERMDISP; p = 0.0398, p = 0.0139, respectively).

2.7. Differentially Abundant zOTUs under Different Feeding Treatments

Multivariate analysis of individual zOTUs using the R package Mvabund revealed that diet and supplement, as well as the interaction between diet and supplement had a significant effect on the bacterial community structure in the faecal samples (Table 6). At the zOTU level, diet had a stronger effect than supplementation with *S. filiforme* on the bacterial community structure by affecting the abundance of 77 zOTUs (6.24% of total zOTUs) (Table 6).

Table 6. Summary of statistical tests on differential zOTU abundance.

Global Test (GLMs) by Mvabund		
Diet: p < 0.0001		
Treatment: p = 0.003		
Diet × Treatment: p < 0.007		
Univariate Analysis by Mvabund (p < 0.05)		
Factor	*Number of differentially abundant OTUs*	*% of total number of OTUs*
Diet	77	6.24%
Treatment	35	0.32%
Total (unique zOTUs affected by one or more factors)	81	6.57%

zOTUs belonging to the phylum Firmicutes (families: Lachnospiraceae, Peptococcaceae and Ruminococcaceae; genus: *Acetatifactor*, *Anaerostipes*, *Blautia*, *GCA-900066575*, *Lachnoclostridium*, *Lachnospiraceae* FCS020 group, *Lachnospiraceae* NK4A136 group, *Lachnospiraceae* UCG-006, *Lachnospiraceae* UCG-008, *Roseburia*, unclassified Lachnospiraceae, unclassified Peptococcaceae, *Butyricicoccus*, *Ruminiclostridium*, *Ruminiclostridium 9* and unclassified Ruminococcaceae) were reduced in abundance or absent in C and CSF rats compared to H and HSF rats, respectively, while zOTUs belonging to the family Ruminococcaceae (genus: *Ruminococcaceae* NK4A214 group) were enriched in C and CSF rats compared to H and HSF rats (Table 7). Bacteria belonging to the phylum Actinobacteria (families: Bifidobacteriaceae and Eggerthellaceae; genus: *Bifidobacterium* and *Enterorhabdus*) and the phylum Bacteroidetes (families: Bacteroidaceae, Muribaculaceae and Prevotellaceae; genus: *Bacteroides*, unclassified Muribaculaceae and *Prevotellaceae* UCG-001) were either reduced or absent in H and HSF rats (Table 7).

Table 7. Relative abundance of zOTUs affected by diet (ANOVA with p adjusted <0.05) between C, CSF, H and HSF rats.

OTU_ID	C (%)	CSF (%)	H (%)	HSF (%)	Phylum	Family	Genus
Zotu42	0.62	0.31	0.00	0.02	Actinobacteria	Bifidobacteriaceae	*Bifidobacterium*
Zotu80	0.29	0.22	0.00	0.00	Actinobacteria	Bifidobacteriaceae	*Bifidobacterium*
Zotu168	0.12	0.09	0.01	0.01	Actinobacteria	Eggerthellaceae	*Enterorhabdus*
Zotu20	0.95	0.90	0.02	0.08	Bacteroidetes	Bacteroidaceae	*Bacteroides*
Zotu21	1.11	0.73	0.16	0.09	Bacteroidetes	Muribaculaceae	unclassified
Zotu27	1.05	0.37	0.05	0.06	Bacteroidetes	Muribaculaceae	unclassified
Zotu79	0.40	0.13	0.02	0.01	Bacteroidetes	Muribaculaceae	unclassified
Zotu541	0.02	0.05	0.00	0.00	Bacteroidetes	Muribaculaceae	unclassified
Zotu857	0.08	0.04	0.00	0.00	Bacteroidetes	Muribaculaceae	unclassified
Zotu978	0.12	0.11	0.01	0.03	Bacteroidetes	Muribaculaceae	unclassified
Zotu1036	0.05	0.02	0.00	0.00	Bacteroidetes	Muribaculaceae	unclassified
Zotu1144	0.12	0.04	0.00	0.01	Bacteroidetes	Muribaculaceae	unclassified
Zotu10	2.06	2.28	0.28	0.19	Bacteroidetes	Prevotellaceae	*Prevotellaceae* UCG-001

Table 7. *Cont.*

OTU_ID	C (%)	CSF (%)	H (%)	HSF (%)	Phylum	Family	Genus
Zotu916	0.00	0.00	0.02	0.02	Firmicutes	Lachnospiraceae	*Acetatifactor*
Zotu77	0.04	0.05	0.30	0.54	Firmicutes	Lachnospiraceae	Anaerostipes
Zotu244	0.01	0.00	0.07	0.11	Firmicutes	Lachnospiraceae	*Blautia*
Zotu49	0.01	0.02	0.81	0.43	Firmicutes	Lachnospiraceae	GCA-900066575
Zotu76	0.03	0.03	0.64	0.20	Firmicutes	Lachnospiraceae	*Lachnoclostridium*
Zotu856	0.00	0.00	0.03	0.02	Firmicutes	Lachnospiraceae	*Lachnospiraceae* FCS020 group
Zotu1061	0.00	0.00	0.03	0.03	Firmicutes	Lachnospiraceae	*Lachnospiraceae* FCS020 group
Zotu37	0.01	0.01	0.81	1.19	Firmicutes	Lachnospiraceae	*Lachnospiraceae* NK4A136 group
Zotu100	0.36	0.14	0.00	0.02	Firmicutes	Lachnospiraceae	*Lachnospiraceae* NK4A136 group
Zotu123	0.00	0.00	0.20	0.40	Firmicutes	Lachnospiraceae	*Lachnospiraceae* NK4A136 group
Zotu182	0.00	0.00	0.18	0.23	Firmicutes	Lachnospiraceae	*Lachnospiraceae* NK4A136 group
Zotu201	0.01	0.01	0.18	0.13	Firmicutes	Lachnospiraceae	*Lachnospiraceae* NK4A136 group
Zotu544	0.00	0.00	0.13	0.09	Firmicutes	Lachnospiraceae	*Lachnospiraceae* NK4A136 group
Zotu561	0.00	0.00	0.06	0.03	Firmicutes	Lachnospiraceae	*Lachnospiraceae* NK4A136 group
Zotu658	0.00	0.00	0.48	0.54	Firmicutes	Lachnospiraceae	*Lachnospiraceae* NK4A136 group
Zotu762	0.00	0.00	0.02	0.04	Firmicutes	Lachnospiraceae	*Lachnospiraceae* NK4A136 group
Zotu847	0.00	0.00	0.03	0.02	Firmicutes	Lachnospiraceae	*Lachnospiraceae* NK4A136 group
Zotu966	0.00	0.00	0.08	0.10	Firmicutes	Lachnospiraceae	*Lachnospiraceae* NK4A136 group
Zotu1157	0.00	0.01	0.12	0.35	Firmicutes	Lachnospiraceae	*Lachnospiraceae* NK4A136 group
Zotu26	0.00	0.00	1.65	1.27	Firmicutes	Lachnospiraceae	*Lachnospiraceae* UGC-006
Zotu110	0.01	0.02	0.49	0.25	Firmicutes	Lachnospiraceae	*Lachnospiraceae* UGC-008
Zotu35	0.01	0.01	1.69	0.32	Firmicutes	Lachnospiraceae	*Roseburia*
Zotu556	0.00	0.00	0.02	0.04	Firmicutes	Lachnospiraceae	*Roseburia*
Zotu582	0.00	0.00	0.02	0.05	Firmicutes	Lachnospiraceae	*Roseburia*
Zotu604	0.00	0.00	0.01	0.05	Firmicutes	Lachnospiraceae	*Roseburia*
Zotu625	0.00	0.00	0.01	0.03	Firmicutes	Lachnospiraceae	*Roseburia*
Zotu634	0.00	0.00	0.01	0.05	Firmicutes	Lachnospiraceae	*Roseburia*
Zotu25	0.00	0.00	1.10	0.83	Firmicutes	Lachnospiraceae	unclassified
Zotu52	0.09	0.04	0.48	0.48	Firmicutes	Lachnospiraceae	unclassified
Zotu73	0.02	0.00	0.24	0.44	Firmicutes	Lachnospiraceae	unclassified
Zotu101	0.00	0.00	0.28	0.24	Firmicutes	Lachnospiraceae	unclassified
Zotu174	0.00	0.00	0.13	0.32	Firmicutes	Lachnospiraceae	unclassified
Zotu197	0.00	0.00	0.46	0.05	Firmicutes	Lachnospiraceae	unclassified
Zotu198	0.02	0.02	0.27	0.32	Firmicutes	Lachnospiraceae	unclassified
Zotu221	0.00	0.00	0.14	0.09	Firmicutes	Lachnospiraceae	unclassified
Zotu226	0.00	0.00	0.15	0.22	Firmicutes	Lachnospiraceae	unclassified
Zotu277	0.00	0.00	0.20	0.04	Firmicutes	Lachnospiraceae	unclassified
Zotu293	0.00	0.00	0.04	0.18	Firmicutes	Lachnospiraceae	unclassified
Zotu516	0.00	0.00	0.07	0.06	Firmicutes	Lachnospiraceae	unclassified
Zotu528	0.00	0.00	0.05	0.03	Firmicutes	Lachnospiraceae	unclassified
Zotu530	0.00	0.00	0.06	0.05	Firmicutes	Lachnospiraceae	unclassified
Zotu590	0.00	0.00	0.05	0.04	Firmicutes	Lachnospiraceae	unclassified
Zotu757	0.00	0.00	0.01	0.04	Firmicutes	Lachnospiraceae	unclassified
Zotu891	0.00	0.00	0.02	0.02	Firmicutes	Lachnospiraceae	unclassified
Zotu937	0.00	0.00	0.07	0.26	Firmicutes	Lachnospiraceae	unclassified
Zotu988	0.00	0.00	0.01	0.01	Firmicutes	Lachnospiraceae	unclassified
Zotu1043	0.01	0.02	0.23	0.17	Firmicutes	Lachnospiraceae	unclassified
Zotu1161	0.00	0.00	0.09	0.04	Firmicutes	Lachnospiraceae	unclassified
Zotu1200	0.00	0.01	0.33	0.16	Firmicutes	Lachnospiraceae	unclassified
Zotu247	0.00	0.00	0.13	0.04	Firmicutes	Peptococcaceae	unclassified
Zotu398	0.05	0.04	0.00	0.00	Firmicutes	Peptococcaceae	unclassified
Zotu384	0.00	0.00	0.10	0.02	Firmicutes	Ruminococcaceae	*Butyricicoccus*
Zotu279	0.00	0.00	0.07	0.08	Firmicutes	Ruminococcaceae	*Ruminiclostridium*
Zotu614	0.00	0.00	0.02	0.04	Firmicutes	Ruminococcaceae	*Ruminiclostridium*
Zotu958	0.00	0.00	0.01	0.01	Firmicutes	Ruminococcaceae	*Ruminiclostridium*
Zotu63	0.00	0.00	0.51	0.30	Firmicutes	Ruminococcaceae	*Ruminiclostridium 9*
Zotu133	0.01	0.00	0.15	0.23	Firmicutes	Ruminococcaceae	*Ruminiclostridium 9*
Zotu135	0.00	0.00	0.17	0.21	Firmicutes	Ruminococcaceae	*Ruminiclostridium 9*
Zotu643	0.01	0.04	0.00	0.00	Firmicutes	Ruminococcaceae	*Ruminiclostridium 9*
Zotu38	0.93	0.21	0.00	0.00	Firmicutes	Ruminococcaceae	*Ruminiclostridium* NK4A214 group
Zotu218	0.08	0.07	0.01	0.01	Firmicutes	Ruminococcaceae	*Ruminiclostridium* NK4A214 group
Zotu50	0.01	0.04	0.50	0.38	Firmicutes	Ruminococcaceae	unclassified
Zotu62	0.02	0.00	0.46	0.43	Firmicutes	Ruminococcaceae	unclassified
Zotu275	0.00	0.00	0.06	0.12	Firmicutes	Ruminococcaceae	unclassified

Differential abundance analysis was performed using Mvabund. C, corn starch diet-fed rats; CSF, corn starch diet-fed rats supplemented with *Sarconema filiforme*; H, high-carbohydrate, high-fat diet-fed rats; HSF, high-carbohydrate, high-fat diet-fed rats supplemented with *Sarconema filiforme*.

A total of four zOTUs (0.32% of total zOTUs) belonging mostly to phylum Firmicutes were significantly affected by *S. filiforme* supplementation (Table 8). One zOTU belonging to the phylum

Bacteroidetes was enriched in C and H rats, while bacteria belonging to the phylum Firmicutes and family Ruminococcaceae (genus: *Ruminococcaceae* UCG-014) and the phylum Proteobacteria and family Desulfovibrionaceae (genus: *Bilophila*) were absent in C and H rats (Table 8).

Table 8. Relative abundance of zOTUs affected by treatment (ANOVA with *p* adjusted <0.05) between C, CSF, H and HSF rats.

OTU_ID	C (%)	CSF (%)	H (%)	HSF (%)	Phylum	Family	Genus
Zotu15	0.72	0.00	3.02	0.00	Bacteroidetes	Muribaculaceae	unclassified
Zotu232	0.00	0.06	0.00	0.13	Firmicutes	Ruminococcaceae	Ruminococcaceae UCG-014
Zotu595	0.00	0.04	0.00	0.01	Firmicutes	Ruminococcaceae	Ruminococcaceae UCG-014
Zotu40	0.00	0.70	0.00	0.43	Proteobacteria	Desulfovibrionaceae	Bilophila

Differential abundance analysis was performed using Mvabund. C, corn starch diet-fed rats; CSF, corn starch diet-fed rats supplemented with *Sarconema filiforme*; H, high-carbohydrate, high-fat diet-fed rats; HSF, high-carbohydrate, high-fat diet-fed rats supplemented with *Sarconema filiforme*.

2.8. Correlation of Microbiota and Physiological Parameters

Combined analysis of bacterial community structure and physiological parameters was performed. The Mantel test revealed that overall the bacterial community structure and the physiological data are correlated (Mantel statistic r = 0.2177; *p* = 0.0071). Table 9 and Figure 12 show how individual physiological parameters contribute to the differences in bacterial community structure between treatments (function envfit – vegan R package).

Table 9. Correlation between bacterial community structure and physiological parameters (*p* < 0.05).

Physiological Variables	R^2	*p*-Value
Epididymal fat	0.59	0.001
Water intake	0.58	0.002
Total abdominal fat	0.56	0.001
Retroperitoneal fat	0.54	0.002
Systolic blood pressure	0.53	0.001
Left ventricle and septum wet weight	0.47	0.001
Fat mass	0.46	0.001
Kidneys wet weight	0.44	0.002
Omental fat	0.40	0.007
Body weight	0.39	0.004
Liver wet weight	0.37	0.011
Right ventricle wet weight	0.33	0.003
Oral glucose tolerance area under the curve	0.33	0.025
Oral glucose tolerance 120-minute blood glucose	0.29	0.027
Food intake	0.25	0.033

Physiological variables were further correlated with individual zOTUs (Table 10). A total of 44 zOTUs were found to be statistically correlated with at least one of the physiological parameters (*p* < 0.05). Of the zOTUs, 33 out of 44 belonged to the phylum Firmicutes, nine zOTUs to the phylum Bacteroidetes, one zOTU to the phylum Proteobacteria and one zOTU to the phylum Actinobacteria. Food intake (7 of 44 zOTUs or 15.91%) was inversely correlated with the relative abundance of the selected zOTU. In contrast, water intake (16 of 44 zOTUs or 36.36%), epididymal fat (4 of 44 zOTUs or 9.1%), left ventricle and septum weight (3 of 44 zOTUs or 6.82%), oral glucose tolerance (3 of 44 zOTUs or 6.82%), systolic blood pressure (3 of 44 zOTUs or 6.82%), liver wet weight (3 of 44 zOTUs or 6.82%) and total abdominal fat (2 of 44 zOTUs or 4.55%) were positively correlated with the selected zOTUs (Table 10).
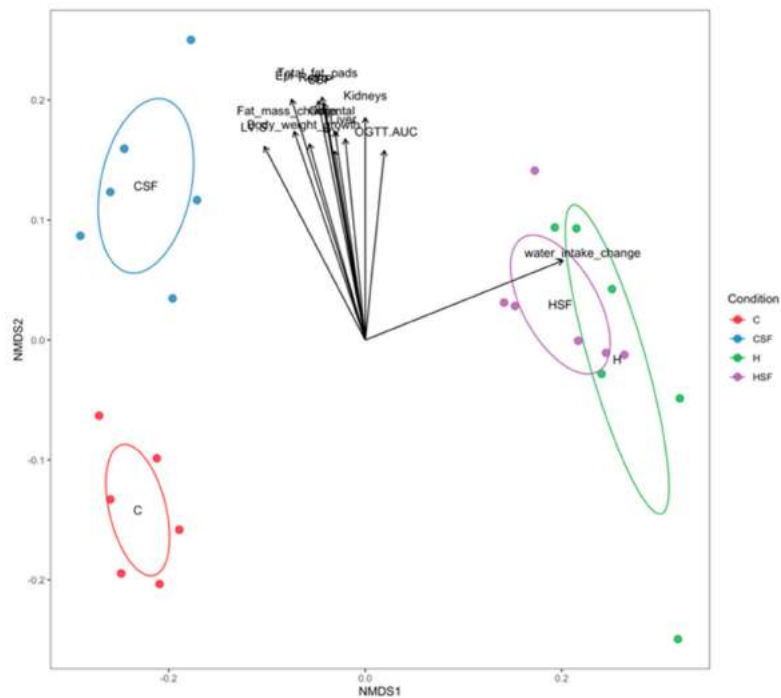
Figure 12. Correlation between bacterial community structure (points) and environmental variables (arrows). C, corn starch diet-fed rats; CSF, corn starch diet-fed rats supplemented with *Sarconema filiforme*; H, high-carbohydrate, high-fat diet-fed rats; HSF, high-carbohydrate, high-fat diet-fed rats supplemented with *Sarconema filiforme*.

Table 10. Taxonomic assignments of the zOTUs strongly correlated with physiological parameters.

OTU_ID	Phylum	Family	Genus	Correlation with Physiological Parameters
Zotu42	Actinobacteria	Bifidobacteriaceae	*Bifidobacterium*	Water intake (−)
Zotu20	Bacteroidetes	Bacteroidaceae	*Bacteroides*	Left ventricle and septum wet weight (+), water intake (−)
Zotu1036	Bacteroidetes	Muribaculaceae	unclassified	Water intake (−)
Zotu1144	Bacteroidetes	Muribaculaceae	unclassified	Water intake (−)
Zotu21	Bacteroidetes	Muribaculaceae	unclassified	Water intake (−)
Zotu27	Bacteroidetes	Muribaculaceae	unclassified	Water intake (−)
Zotu541	Bacteroidetes	Muribaculaceae	unclassified	Epididymal fat (+)
Zotu79	Bacteroidetes	Muribaculaceae	unclassified	Water intake (−)
Zotu857	Bacteroidetes	Muribaculaceae	unclassified	Water intake (−)
Zotu10	Bacteroidetes	Prevotellaceae	*Prevotellaceae* UCG-001	Left ventricle and septum wet weight (+), water intake (−)
Zotu77	Firmicutes	Lachnospiraceae	*Anaerostipes*	Food intake (−), water intake (+)
Zotu244	Firmicutes	Lachnospiraceae	*Blautia*	Water intake (+)
Zotu856	Firmicutes	Lachnospiraceae	*Lachnospiraceae* FCS020 group	Water intake (+)
Zotu100	Firmicutes	Lachnospiraceae	*Lachnospiraceae* NK4A136 group	Water intake (−)
Zotu37	Firmicutes	Lachnospiraceae	*Lachnospiraceae* NK4A136 group	Left ventricle and septum wet weight (−)
Zotu762	Firmicutes	Lachnospiraceae	*Lachnospiraceae* NK4A136 group	Water intake (+)
Zotu556	Firmicutes	Lachnospiraceae	*Roseburia*	Food intake (−), water intake (+)
Zotu582	Firmicutes	Lachnospiraceae	*Roseburia*	Food intake (−)
Zotu604	Firmicutes	Lachnospiraceae	*Roseburia*	Food intake (−)
Zotu625	Firmicutes	Lachnospiraceae	*Roseburia*	Water intake (+)
Zotu634	Firmicutes	Lachnospiraceae	*Roseburia*	Food intake (−)
Zotu101	Firmicutes	Lachnospiraceae	unclassified	Water intake (−)
Zotu174	Firmicutes	Lachnospiraceae	unclassified	Food intake (−)
Zotu198	Firmicutes	Lachnospiraceae	unclassified	Left ventricle and septum wet weight (−)
Zotu25	Firmicutes	Lachnospiraceae	unclassified	Water intake (+)

Table 10. *Cont.*

OTU_ID	Phylum	Family	Genus	Correlation with Physiological Parameters
Zotu516	Firmicutes	Lachnospiraceae	unclassified	Water intake (+)
Zotu52	Firmicutes	Lachnospiraceae	unclassified	Left ventricle and septum wet weight (−)
Zotu530	Firmicutes	Lachnospiraceae	unclassified	Water intake (+)
Zotu590	Firmicutes	Lachnospiraceae	unclassified	Left ventricle and septum wet weight (−)
Zotu73	Firmicutes	Lachnospiraceae	unclassified	Water intake (+)
Zotu757	Firmicutes	Lachnospiraceae	unclassified	Food intake (−)
Zotu891	Firmicutes	Lachnospiraceae	unclassified	Water intake (+)
Zotu988	Firmicutes	Lachnospiraceae	unclassified	Water intake (+)
Zotu398	Firmicutes	Peptococcaceae	unclassified	Water intake (−)
Zotu279	Firmicutes	Ruminococcaceae	*Ruminiclostridium*	Left ventricle and septum wet weight (−), water intake (+)
Zotu614	Firmicutes	Ruminococcaceae	*Ruminiclostridium*	Left ventricle and septum wet weight (−)
Zotu643	Firmicutes	Ruminococcaceae	*Ruminiclostridium 9*	Body weight (+), retroperitoneal fat (+), epididymal fat (+), omental fat (+), Total abdominal fat (+), fat mass (+), Liver weight (+), Left ventricle and septum wet weight (+), oral glucose tolerance 120-minute blood glucose (+), systolic blood pressure (+), water intake (−)
Zotu133	Firmicutes	Ruminococcaceae	*Ruminiclostridium 9*	Water intake (+)
Zotu135	Firmicutes	Ruminococcaceae	*Ruminiclostridium 9*	Water intake (+)
Zotu63	Firmicutes	Ruminococcaceae	*Ruminiclostridium 9*	Left ventricle and septum wet weight (−)
Zotu595	Firmicutes	Ruminococcaceae	*Ruminococcaceae* UCG-014	Epididymal fat (+), retroperitoneal fat (+), right ventricle wet weight (+), systolic blood pressure (+)
Zotu232	Firmicutes	Ruminococcaceae	*Ruminococcaceae* UCG-014	Liver wet weight (+)
Zotu62	Firmicutes	Ruminococcaceae	unclassified	Left ventricle and septum wet weight (−), water intake (+)
Zotu40	Proteobacteria	Desulfovibrionaceae	*Bilophila*	Epididymal fat (+), omental fat (+), retroperitoneal fat (+), total abdominal fat (+), kidney wet weight (+), liver wet weight (+), oral glucose tolerance 120-minute blood glucose (+), oral glucose tolerance area under the curve (+), systolic blood pressure (+)

Differential abundance analysis was performed using Mvabund. This table includes the physiological parameters strongly correlated *(P < 0.05)* with the bacterial community and incorporates zOTUs that interact with at least 1 of these parameters. Plus sign (+) indicates positive correlations, while minus sign (−) indicates negative correlations.

The relative abundances of zOTUs belonging to the phylum Firmicutes and the families Lachnospiraceae (genus: *Anaerostipes*, *Blautia*, *Lachnospiraceae* FCS020 group, *Lachnospiraceae* NK4A136 group, *Roseburia*, unclassified Lachnospiraceae: zOTU77, zOTU244, zOTU856, zOTU762, zOTU37, zOTU556, zOTU582, 174, zOTU25, zOTU198, zOTU52 and zOTU590) and Ruminococcaceae (genus: *Rumiclostridium* and *Rumiclostridium 9*: zOTU279, zOTU614, zOTU133, zOTU135 and zOTU63) were inversely correlated with food intake and left ventricle and septum weight and positively correlated with water intake. While bacteria belonging to the phylum Firmicutes and family Peptococcaceae (zOTU398) were negatively correlated to water intake, bacteria belonging to the family Ruminococcaceae UCG-014 (zOTU595, zOTU232) were positively correlated to several physiological parameters including epididymal fat, retroperitoneal fat, right ventricle weight, systolic blood pressure and liver wet weight.

The relative abundances of bacteria belonging to the phylum Actinobacteria and family Bifidobacteriaceae; the phylum Bacteroidetes and families Bacteroidaceae, Muribaculaceae and Prevotellaceae (for example: zOTU42, zOTU1036, zOTU1144, zOTU21, zOTU27, zOTU79 and zOTU857) were negatively correlated to water intake. Several zOTUs belonging to the phylum Bacteroidetes and families Bacteroidaceae and Prevotellaceae were also positively correlated with left ventricle and septum weight (zOTU20 and zOTU10). The relative abundance of bacteria belonging to the phylum Proteobacteria and family Desulfovibrionaceae was positively correlated to epididymal fat, kidney weight, liver weight, oral glucose tolerance 120-minute blood glucose, oral glucose tolerance area under the curve, omental fat, retroperitoneal fat, systolic blood pressure and total abdominal fat (zOTU40).

3. Discussion

This project demonstrates that local Australian cultivation of *S. filiforme* produced significant and reliable yields of biomass in intensive tank-based culture, which can therefore potentially be a source of commercial quantities of ι-carrageenan. Australia has unique and untapped seaweed resources [11]; however, as of 2014, the Australian seaweed industry was small and only based on the harvest of stormcast kelp for alginate and fertiliser and on introduced species of *Undaria* for the extraction of bioactive compounds [12]. However, indigenous species such as *S. filiforme* could supply high-value fresh and dried foods as well as compounds for the nutraceutical and pharmaceutical markets. Consuming locally grown foods is considered important, because it reduces transport to markets and environmental impact to decrease CO_2 emissions. In addition, local production supports the local economy as evidenced by the successful commercial cultivation of the red seaweed, *K. alvarezii*, in countries such as the Philippines and Indonesia as a main source of carrageenan for the food industry for more than 40 years [13].

Further, we show that whole dried *S. filiforme* may be useful in reversing metabolic syndrome. Metabolic syndrome including abdominal obesity, hypertension, hyperglycaemia, fatty liver and inflammation increases the risk of cardiovascular disease and diabetes; this syndrome is mimicked by a diet high in simple sugars, saturated and *trans* fats in rats [14]. This validated dietary model of human metabolic syndrome has been previously reported to show reversal of changes by interventions with seaweeds [15,16]. The major findings from the current reversal study were that *S. filiforme* decreased metabolic, cardiovascular and liver changes by 9-40% in obese rats fed a high-carbohydrate, high-fat diet, including some variables that were effectively reversed including liver enzymes and systolic blood pressure. These results are consistent with our previous study where *K. alvarezii* containing κ-carrageenan was used in a prevention protocol [8]. The soluble fibre content, which also approximates the carrageenan content, was ~35% in *K. alvarezii* and ~12% in *S. filiforme*, giving a soluble fibre content in the rat diets of around 1.7% and 0.5%, respectively. This was markedly less than the upper limit of 5% to avoid any safety risks, as recommended by the Scientific Committee on Food of the European Commission, for carrageenans below a molecular weight limit of 50 kDa when used as a food additive [17]. Although carrageenans have been used in subcutaneous injections to induce rat paw oedema as a model of inflammation [18], there have been dietary studies with no adverse effects using 5% ι-carrageenan prepared from *E. denticulatum* (previously *E. spinosum*) [19,20], although systemic administration of carrageenan increased biliary antibody titre, which is suggestive of bacterial intrusion [20,21]. In the literature, there are conflicting reports on the effects of carrageenans on the gastrointestinal tract, although the confusion may be in part due to inconsistent nomenclature. Degraded carrageenans have been incorrectly referred to as poligeenans, which are not produced biologically. Poligeenans are produced in the laboratory or commercially by subjecting carrageenan to very low pH at 0.9–1.3 and non-physiological temperatures of >80°C for several hours [22]. As carrageenans are not absorbed from the gastrointestinal tract after oral administration, studies using systemic administration are not appropriate for a risk assessment of carrageenans when used as food products in subjects without gastrointestinal pathology [23]. However, oral studies can determine

local gastrointestinal risk; the current study shows no histopathological impact on the ileum or colon in H rats compared to HSF rats, consistent with the previous study on oral administration of *K. alvarezii* containing κ-carrageenan [8].

Traditionally, people in East Asian countries, such as Korea, Japan and China, consume more seaweeds as food and ingredients of traditional medicine than other populations [24]. The average intake of seaweeds in Japan was 14.3 g (wet weight)/day [25]. In the current study, the dose in rats equates to approximately 6.3 g of seaweed/day for humans [10]. Therefore, it is realistic that this quantity can be consumed as a single seaweed such as *S. filiforme* in the usual diet to translate its health benefits. Further, production of therapeutic amounts of this seaweed requires a small area, as the 42 m^2 research facility of the University of the Sunshine Coast could provide enough seaweed for 179 people to be treated continuously, based on measured seaweed growth rates. This is markedly more efficient usage of space than with other sources of dietary fibre such as cereals; as an example, the average cereal yield in the USA was 828 g/m^2/year in 2017 [26] compared to the average yield of seaweed at the University of the Sunshine Coast of around 189 g/m^2/week (or around 10 kg/m^2/year). Scaling up to produce commercial amounts of seaweed is therefore realistic.

Dietary fibre is classified as either soluble (non-cellulosic, polysaccharides, oligosaccharides, pectins, β-glucans and gums) or insoluble (cellulose, hemicellulose and lignin). The major physiological effects of soluble fibre are delayed gastric emptying, regulation of blood glucose levels and lowered serum cholesterol concentrations, due to increasing gut content viscosity and colonic fermentation. In contrast, the major effects of insoluble fibre are shortened gut transit time and improved laxation, both due to faecal bulking capacity and support for the growth of intestinal bacteria due to colonic fermentation [27]. *S. filiforme* contains about 22% fibre, which equates to 1.3 g of fibre per day if the human dose is 6.3 g/day, based on the Reagan-Shaw rat to human scaling equation [10]. The American Dietetic Association recommended a daily dietary fibre intake of 25 g for adult females and 38 g for adult males [28]. Therefore, *Sarconema* at this dose would increase fibre consumption, but alone would not provide sufficient fibre to meet this recommendation; consequently, other fibre-rich foods such as fruits, vegetables, beans and grains would be necessary components of the diet. Epidemiological and clinical studies have demonstrated that dietary fibre intake is inversely related to obesity [29], type 2 diabetes [30] and cardiovascular disease [31]. The consumption of dietary fibre likely reduces body fat accumulation due to several factors such as fermentation of the fibre in the colon, stimulating production of glucagon-like-peptide-1 and peptide YY [32]. These gut hormones increase satiety, which may lead to decreased meal frequency and size. Consumption of insoluble fibre reduced body weight and body fat and also normalised fasting glucose and insulin concentrations in overweight and obese adults [33].

Carrageenans undergo minimal digestion in the stomach and are then fermented by colonic bacteria [34], hence meeting the definition of prebiotics [35]. Health benefits of prebiotics include decreased blood pressure and body weight [27,36] similar to the responses from the current study. Using the same rat model of diet-induced obesity, a prebiotic mixture of inulin and oligofructose was reported as an effective dietary fibre, reducing body weight, plasma concentrations of free fatty acids and triglycerides, and systolic blood pressure and attenuating inflammatory cell infiltration in the heart and liver [37]. Red seaweeds show responses that suggest the biological actions of fibre as prebiotics. Subjects supplemented with the red seaweed *Chondrus crispus* showed improved gut health and immune modulation [38]. Another red seaweed, *Mastocarpus stellatus*, with 31.7% dietary fibre reduced triglycerides and total cholesterol concentrations, however, there was no effect on body weight in healthy Wistar rats [39]. Complex polysaccharides exert their action through a wide range of mechanisms including selective fermentation, lowering the gut pH, faecal bulking, preventing gut colonisation by pathogens, controlling putrefactive bacteria, and therefore reducing the host's exposure to toxic metabolites [40]. These effects are likely due to dietary fibre increasing short-chain fatty acid production as these are used as an energy source by selected gut microbiota. Short-chain fatty acids

decrease the luminal pH, improve calcium and magnesium absorption, reduce potential pathogenic bacteria and act as an energy source for epithelial cells [41].

4. Materials and Methods

4.1. Sarconema filiforme *Source and Analysis*

S. filiforme was cultured at the University of the Sunshine Coast seaweed aquaculture facility at the Bribie Island Research Centre, Woorim, QLD, Australia (27°04′10″ S; 153°12′15″ E) from January to March 2018. Production (yield of dry weight/m^2/day) was measured through weekly harvests from up to five 1000 L fibreglass tanks over 13 weeks from cultures initially stocked each week at, on average, 7 g fresh weight per 1000 L of seawater. Cultures received flow-through seawater with weekly addition of nutrients (MAF, Manutech, Cavan, SA, Australia). Fresh samples were harvested each week, dehydrated and a total of 3 kg dried and milled *S. filiforme* was sub-sampled across the three months of production and stored in vacuum-sealed bags containing silica desiccant. Compositional analysis of the seaweed was performed as detailed previously [42,43].

Attenuated Total Reflectance-Fourier-Transform Infrared Spectroscopy (ATR-FTIR) was performed at Southern Cross University, Lismore, NSW, Australia to determine carrageenan subtypes in *S. filiforme* and *K. alvarezii* as a control and commercial grade ι-carrageenan and κ-carrageenan (Sigma-Aldrich Australia, Castle Hill, NSW, Australia) as standards. The transmittance spectra were recorded from 2000 to 675 cm^{-1} [44].

4.2. Rats and Diets

All experimental protocols were approved by the Animal Ethics Committee of the University of Southern Queensland under the guidelines of the National Health and Medical Research Council of Australia. Male Wistar rats (8–9 weeks old; 338 ± 1 g, n = 48) were obtained from the Animal Resource Centre, Murdoch, WA, Australia. Rats were individually housed in a temperature-controlled (21 ± 2 °C), 12-hour light/dark conditions with free access to food and water. Rats were randomly distributed into four groups, each of 12 rats. Two groups were fed either corn starch or high-carbohydrate, high-fat diets (C and H, respectively) [14] for a full 16 weeks. The other two groups received C and H diets for the first eight weeks and then received 5% *S. filiforme* in the diet for the last eight weeks (CSF and HSF, respectively). The C diet contained 570 g of cornstarch, 155 g of powdered rat food (Specialty Feeds, Glen Forest, WA, Australia), 25 g of Hubble, Mendel and Wakeman salt mixture (MP Biomedicals, Seven Hills, NSW, Australia), and 250 g of water per kilogram of diet. The H diet contained 175 g of fructose, 395 g of sweetened condensed milk, 200 g of beef tallow, 155 g of powdered rat food (all obtained from local food suppliers and supermarkets), 25 g of Hubble, Mendel and Wakeman salt mixture and 50 g of water per kilogram of diet. In addition, the drinking water for the H and HSF groups was supplemented with 25% fructose [14].

4.3. Rat Measurements

Dual-energy X-ray absorptiometry, non-invasive systolic blood pressure, abdominal circumference, oral glucose and insulin tolerance tests and indirect calorimetry were performed as described [14]. Euthanasia followed by heparin injection, blood collection, centrifugation, storage and then isolated Langendorff heart preparation and measurements, plasma measurements, organ weights, organ bath study and histological analyses were performed as described [14].

4.4. Gut Microbiota Analysis

Immediately after euthanasia and organ removal, two or three faecal pellets were collected from the colon of rats and stored at −80 °C in nuclease-free tubes. Total microbial community DNA was extracted from faecal samples using the DNeasy Powersoil Kit (Qiagen Australia, Chadstone, VIC, Australia) following the manufacturer's instructions [45]. The bacterial gut

microbiota was then characterised by amplifying and sequencing the 16S rRNA gene. The primers, 341F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and 785R (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) were used to amplify the V3-V4 regions of the 16S rRNA gene, which was then sequenced on an Illumina MiSeq platform. Sequencing reads were processed to form zOTUs, which were taxonomically classified against the SILVA database.

The reaction mixture (50 μL total volume per sample) to amplify the 16S rRNA gene consisted of Econotaq®PLUS GREEN 2× Master Mix (Astral Scientific, Gymea, NSW, Australia) (25 uL), Ambion®nuclease-free water (17 μL), the primer pair 341F and 785R (1.5 μL of each; 10 μM) and DNA template (5 μL). The PCR program consisted of an initial denaturation at 94 °C (2 min), followed by 35 cycles of denaturation at 94ºC (30 s), annealing at 55ºC (30 s), extension at 72 °C (40 s) and a final extension at 72 °C (7 min). PCR products were then quantified using gel electrophoresis. Paired-end sequencing (2 × 300 bp) of the resulting 16S rRNA gene amplicons was performed at the Ramaciotti Centre for Genomics, University of New South Wales on an Illumina MiSeq platform following the MiSeq System User Guide [46]. For 16S rRNA gene sequencing analysis, sequence data were initially quality-filtered and trimmed using Trimmomatic version 0.36 truncating reads if the quality dropped below 20 in a sliding window of 4 bp [47]. USEARCH version 11.0.667 [48] was used for further processing [49] to merge and quality-filter sequencing reads, excluding reads with < 250 or > 550 nucleotides, in addition to reads with more than one ambiguous base or an expected error of more than 1. Filtered sequences were denoised and clustered into unique sequences (zero-radius operational taxonomic units; zOTUs) using the UNOISE algorithm [50] implemented in USEARCH. zOTUs represent unique bacterial entities and roughly are equivalent to species or strains. Chimeric sequences were removed *de novo* during clustering and subsequently in reference mode using UCHIME [51] with the SILVA database (https://www.arb-silva.de/browser/) (SILVA SSURef 132 NR) as a reference [52]. zOTUs were then taxonomically classified (i.e., assigned a likely taxonomic name) by BLASTN [53] against the SILVA database. All non-bacterial zOTUs were removed along with non-BLAST aligned and singleton zOTUs. Finally, processed sequences were mapped on zOTU sequences to calculate the distribution and counts of each zOTU in every sample. Only zOTUs occurring in more than two samples were considered for further statistical analysis.

4.5. Statistical Analysis

Physiological and metabolic data are presented as mean ± standard error of the mean (SEM). These results were tested for variance using Bartlett's test and variables that were not normally distributed were transformed using log10 function prior to statistical analyses. Data from the four groups were tested by two-way analysis of variance. When the interactions and/or the main effects were significant, means were compared using the Newman-Keuls multiple comparison *post hoc* test. Where transformations did not result in normality or constant variance, a Kruskal-Wallis non-parametric test was performed. A *p* value of < 0.05 was considered as statistically significant. All statistical analyses were performed using Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

For microbiota results, rarefaction curves were generated using the *rarecurve* function in *vegan* [54] and used to determine if a complete representation of the sample's microbiota had been achieved given the sequencing effort. Prior to further analysis, the numbers of sequences were standardised across samples to account for different sequencing depths by randomly subsampling each sample to the lowest number of sequences counts obtained for any given sample (i.e., 19,706 counts). Bacterial alpha-diversities (zOTU richness and Shannon's diversity) were calculated in R (version 3.5.3) using the rrarefy function in the vegan package [55]. A one-way analysis of variance test in GraphPad Prism 8.0.2 (San Diego, CA, USA) followed by Tukey's pairwise comparisons test was used to determine the significance between the different groups and a *p* value of <0.05 was considered to be significant.

For multivariate analysis of bacterial communities, zOTU tables were imported into PRIMER [55] to compare the community structure (i.e. relative abundance data). Bray-Curtis similarity coefficients

were calculated using square-root transformed zOTU abundances and the resulting similarity matrix was visualised using non-metric, multi-dimensional scaling (nMDS). Permutational multivariate analysis of variance (PERMANOVA) [56] with 9,999 random permutations was used to test the effect of treatment on bacterial communities in rat faecal samples.

5. Conclusions

Rats fed a high-carbohydrate, high-fat diet supplemented with *S. filiforme* as a source of *ι*-carrageenan decreased body weight, systolic blood pressure, abdominal and liver fat and plasma total cholesterol concentrations compared to H controls. These results are comparable to our previous study with *K. alvarezii* as a source of *κ*-carrageenan, providing evidence that red seaweeds contain compounds such as sulfated polysaccharides (carrageenans) which attenuate symptoms of diet-induced metabolic syndrome in rats. The correlations between changes in the gut microbiota and physiological changes following administration of *S. filiforme* suggests that the mechanism is likely through carrageenans acting as prebiotics as well as systemic anti-inflammatory responses in organs such as heart and liver. Further studies with mechanistic analyses will be valuable to determine the actions of carrageenans *in vivo* that are responsible for their health benefits.

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CHAPTER 4. BROWN SEAWEEDS IN DIET-INDUCED METABOLIC SYNDROME

The single largest crop grown by mariculture in the world is the kelp, *Saccharina japonica* (http://www.seaweed.ie/uses_general/alginates.php), as a source of alginates. Alginates are also part of the cell wall of *Sargassum* species, which can grow rapidly as an invasive species, for example to constitute the world's largest seaweed bloom between West Africa and the America of 8,550 kilometres containing over 20 million tonnes of biomass as of June, 2018 (https://www.bbc.com/news/science-environment-48869100). However, *Sargassum* species can also be grown in controlled conditions for industrial and health products. The traditional uses of *Sargassum* species include as fertiliser, animal feed and for alginate extraction which can be used as polymers for health uses such as wound dressings.

The previous chapters of this thesis have shown that the carrageenans, sulphated polysaccharides from *K. alvarezii* and *S. filiforme*, can prevent or reverse signs of diet-induced metabolic syndrome in rats. This chapter investigated the use of the brown seaweed, *Sargassum siliquosum*, obtained from tropic waters near Townsville in north Queensland, as a functional food. *S. siliquosum* contains alginates and laminarans as non-sulphated linear polysaccharides, and fucoidans as sulphated polysaccharides. *S. siliquosum* was tested in a reversal model of diet-induced metabolic syndrome in rats. There are limited studies on the health benefits of the *Sargassum* species in humans. Traditional Chinese Medicine studies have focused on *Sargassum* species to treat thyroid disease due to its high iodine content. There have been some studies with reported actions against diabetes, liver disease and obesity, as disease states of metabolic syndrome, although these used extracts rather than as a whole food. If the current chapter shows that whole *S. siliquosum* supplementation can improve symptoms in rats with metabolic syndrome, this evidence will provide support for *S. siliquosum* to be part of Australia's seaweed industry, as Sargassum covers large areas off the coast of North Queensland.

Dried brown seaweed Sargassum siliquosum as an intervention for diet-induced obesity in male Wistar rats

Ryan du Preez [1,2], Marie Magnusson [3], Rocky de Nys [4], Marwan Majzoub [5], Torsten Thomas [5], Christina Praeger [6], Giuseppe C. Zuccarello [3], Lindsay Brown [1,2], Sunil K Panchal [2,]

[1]Functional Foods Research Group, University of Southern Queensland, Toowoomba, QLD 4350, Australia

[2]School of Health and Wellbeing, University of Southern Queensland, Toowoomba, QLD 4350, Australia

[3]Environmental Research Institute, Coastal Marine Field Station, University of Waikato, Tauranga 3112, New Zealand

[4]MACRO – The Centre for Macroalgal Resources and Biotechnology, College of Marine and Environmental Sciences, James Cook University, Townsville, QLD 4811, Australia

[5]Centre for Marine Bio-Innovation, University of New South Wales, Sydney, NSW 2052, Australia

[6]School of Biological Sciences, Victoria University of Wellington, Wellington 6140, New Zealand

*Correspondence: Sunil.Panchal@usq.edu.au; Tel.: +61 7 4631 1012

Abstract

Sargassum is an abundant species of brown seaweed containing a mixture of polysaccharides including alginates, fucoidans and laminarans with fucoxanthin as a brown pigment. *Sargassum* can have health benefits when grown in a controlled environment, despite the widespread reports as an invasive species. The therapeutic potential of this seaweed in a rat model of metabolic syndrome has been tested in this study. Male Wistar rats (n = 48) were divided into four groups in a 16 week protocol: C rats were fed a corn starch diet; CS rats were fed a corn starch-based diet for eight weeks, then supplemented with 5% *S. siliquosum* for the next eight weeks; H rats were fed a high-carbohydrate, high-fat diet with increased fructose and sucrose, together with increased saturated and *trans* fats; HS rats were fed this high-carbohydrate, high-fat diet for eight weeks, then supplemented with 5% *S. siliquosum* for the next eight weeks. H rats developed obesity, hypertension, dyslipidaemia, glucose intolerance, fatty liver disease and increased left ventricular collagen deposition. In HS rats compared to H rats, *S. siliquosum* supplementation decreased body weight (C, 388±10; CS, 384±10; H, 547±14; HS, 490±16 g), fat mass, abdominal fat depositon and reduced liver fat vacuole size. There were no changes in systolic blood pressure, left ventricular diastolic stiffness constant, plasma total cholesterol, plasma triglycerides and glucose or insulin tolerance. Thus, *S. siliquosum* containing alginates and fucoidans as fibre improves some metabolic symptoms of diet-induced metabolic syndrome in rats. The most likely mechanism of action is fibre-related slowed nutrient absorption in the small intestine.

Keywords: *Sargassum siliquosum*; brown seaweed; fucoidans; alginates; gut microbiome; metabolic syndrome

1. Introduction

Brown seaweeds such as *Sargassum* species are major sources of polysaccharides for their nutritional and commercial uses, traditionally as fertiliser, animal feed and for alginate extraction (1, 2). The primary polysaccharides of brown seaweeds include alginates, laminarins, fucoidans and cellulose (3); the colour of brown seaweeds is due to fucoxanthin, a xanthophyll pigment. The *Sargassum* genus contains approximately 400 species (4, 5) that inhabit the pelagic zone in widespread locations including the Caribbean, Gulf of Mexico and West Africa (6). In 2011, *Sargassum* species, primarily *S. natans* and *S. fluitans*, received extensive and negative media reports after washing ashore in these areas covering beaches by more than a metre of seaweed (7). However, *Sargassum* species are also grown in controlled environments, for the production of pharmaceuticals (8) and fertilisers (9). *Sargassum* species produce compounds for protection against environmental factors such as heat, pollution, stress, decreased oxygen concentration and ultraviolet radiation (10). While agri-industrial by-products of seaweeds (including *Sargassum*) represent an inexpensive, abundant and sustainable resource, they can have multiple uses including as foods or for health benefits. *Sargassum* species are consumed fresh, after frying or boiling (11), cooked in coconut milk or smoked-dried in Polynesia, China and Japan (12). Consuming *Sargassum* species as a functional food could produce anti-oxidant responses such as nitric oxide scavenging (13) and anti-inflammatory changes such as reduced oedema in carrageenan-induced paw oedema (14).

The full potential of *Sargassum* as a functional food has not been realised. Traditional Chinese Medicine has focused on thyroid disease as the major health benefit from *Sargassum* due to its high iodine content (15). Other disease targets of *Sargassum* could include diabetes as solvent fractions of *S. wightii* inhibited α-amylase and α-glucosidase resulting in delayed glucose absorption and thus lowering of postprandial hyperglycaemia (16). Liver disease may also be a therapeutic target as oral pre-treatment with alcoholic extract of *S. polycystum* (200 mg/kg body weight/day for 15 days) protected the liver against acetaminophen-induced damage (17). There is considerable potential for the use of *Sargassum* products as therapeutic interventions against metabolic diseases (3). One example is fucoxanthin to reduce obesity through expression of uncoupling protein-1; in a human trial 2.4 mg/day of fucoxanthin for 16 weeks reduced body weight by 5.5 kg (18). Importantly,

fucoxanthin concentrations in several brown seaweed lipids are sufficient to show physiological activity when added to general foods (19).

Polysaccharides including fucoidans, laminarins and alginates, and polyphenols including phenolic acids, flavonoids, phlorotannin, stilbenes and lignans from seaweeds have a role in controlling digestion (20). These components reduced the activity of digestive enzymes, modulating enzymes such as α-amylase, α-glucosidase, pepsin and lipase and hence may be used in the treatment of obesity (20). Laminarins as nutraceutical ingredients should be further investigated due to their dietary fibre and anti-oxidant properties (21).

The aim of this study was to determine whether cardiovascular and metabolic health benefits are possible following chronic consumption of *S. siliquosum* as a source of fucoidan, alginates and fucoxanthin. *S. siliquosum* is found predominantly in the Sargasso Sea (western North Atlantic Ocean) and the Coral Sea (off the north-east coast of Australia). We investigated this species as it may be both amenable to cultivation in tropical areas (22) and have cardiovascular responses that are useful in metabolic syndrome (23). Genetic barcoding was used to define the characteristics of *Sargassum* and identifiy *S. siliquosum*. We tested the seaweed using a validated diet-induced rat model of metabolic syndrome that closely mimics the symptoms of human metabolic syndrome. We measured systolic blood pressure, left ventricular diastolic stiffness, changes in thoracic aorta reactivity, cardiac inflammatory cells and collagen deposition in the heart for cardiovascular effects; plasma liver enzyme activities, inflammatory cells and fat vacuoles in the liver; body weight, total cholesterol and triglyceride concentrations and glucose and insulin tolerance tests for metabolic effects. Further, as functional foods may reverse obesity-induced changes in the gut microbiome (24, 25), we characterised the changes in its composition after seaweed treatment. We hypothesised that 5% *S. siliquosum* supplementation for the last 8 weeks of the protocol will reverse the changes induced by the high-carbohydrate, high-fat diet. The mechanisms of these effects could include the actions of fucoxanthin as well as of fucoidan and alginates as prebiotics in the colon by reducing intestinal absorption of carbohydrates and fats (19). The combination of these compounds in the dried seaweed may also prevent infiltration of inflammatory cells into organs such as the heart and liver.

2. Materials and Methods

2.1 Sargassum siliquosum biomass collection

Sargassum siliquosum was collected by snorkel from a rocky reef at approximately 2 m depth in Nelly Bay, Magnetic Island, QLD, Australia (19.1708° S, 146.8471° E), on 28th November 2017, and immediately transported to James Cook University, Townsville, QLD, Australia. Twelve specimens with intact holdfasts and fresh new growth were photographed and selected for genetic barcoding. The remaining fresh biomass was briefly rinsed in batches in filtered seawater followed by freshwater to remove sand, debris, invertebrates and epiphytes. The rinsed biomass was dried at 60°C for 48 h, sorted to remove remaining foreign matter such as coral rubble in holdfasts, milled to 1 mm and homogenised. The biomass was stored in vacuum-sealed bags containing silica desiccant at 4°C until processing or experiments.

2.2 Genetic barcoding

One blade from each of the twelve specimens selected for molecular barcoding was excised and cleaned in autoclaved seawater using a soft paintbrush to remove debris and epiphytes. Each blade was dried using paper towel to remove excess water and subsequently placed in individually labelled zip-lock bags with 50 g of silica beads to desiccate. Dried samples were sent to Victoria University of Wellington, New Zealand, for DNA extraction and sequencing. DNA was extracted using a modified CTAB protocol (26) and the molecular marker mitochondrial gene cytochrome oxidase I (COI) was used to obtain sequences to assign *Sargassum* specimens to a genetic species group. The forward primer GazF2 (50CCAACCAYAAAGATATWGGTAC 30) and reverse primer GazR2 (50GGATGACCAAARAACCAAAA 30) were used (27). Maximum likelihood (ML) phylogenetic trees were constructed in Iqtree with sequences downloaded from Genbank.

2.3 Compositional analyses

All analyses were performed on five subsamples (n = 5) of milled and homogenised material except for amino acid analysis (n = 3) and fibre analysis (n = 2). Moisture content of the dried milled seaweed was quantified using an MS-70 Moisture Analyzer (A&D Company Ltd., Thebarton, SA, Australia) and the content of ash was quantified by weighing the same biomass samples before and after combustion in air at 550°C for 6 h (SEM muffle furnace, LabTek, Brendale, QLD,

Australia). Total lipids and fatty acids were quantified (28), while amino acids were quantified using the Water AccQTag method at the Australian Proteome Analysis Facility (Macquarie Park, NSW, Australia). Protein was taken as the sum of amino acids, and carbohydrate was determined by difference as 100% - (ash + moisture + lipid + protein)%. Total, soluble and insoluble fibre were analysed at Aegic Australia (Australian Export Grains Innovation Centre, North Ryde, NSW, Australia), using an enzymatic-gravimetric method (AOAC 985.29, AOAC 993.19). The content of carbon, hydrogen, nitrogen, sulphur and iodine was quantified using an elemental analyser (OEA laboratories, Callington, UK). Trace elements, metals and metalloids were analysed by ICP-OES and ICP-MS, respectively, at the Advanced Analytical Centre, James Cook University, Townsville, QLD, Australia.

2.4 Rats and diets

All experimental protocols were approved by the Animal Ethics Committee of the University of Southern Queensland (approval number 18REA001) under the guidelines of the National Health and Medical Research Council of Australia. Male Wistar rats (8-9 weeks old; 336 ± 2 g, n = 48) were obtained from the Animal Resource Centre, Murdoch, WA, Australia. Rats were individually housed in a temperature-controlled ($21\pm2°C$), 12-hour light/dark conditions with free access to food and water. Rats were randomly distributed into four groups, each of 12 rats. Two groups fed either corn starch or high-carbohydrate, high-fat diets (C and H, respectively) (29) received the diet for the full 16 weeks. The other two groups received C and H diets for eight weeks and then received C or H diet supplemented with 5% *S. siliquosum* for the final eight weeks (CS and HS, respectively).

2.5 Measurements before euthanasia

A mediquip anaesthesia machine was used to induce light sedation to immobilise the rat. Rats were pre-oxygenated with 2 L/min for three minutes prior to 5% isoflurane induction vaporised in 1.5 L/min. Rats were placed in a nose mask and maintained with 1.5% isoflurane in 1.5 L/min O_2 for the duration of each procedure. Systolic blood pressure measurements were performed on all rats after 8 and 16 weeks of feeding using an MLT1010 Piezo-Electric Pulse Transducer (ADInstruments, Bella Vista, NSW, Australia) and an inflatable tail-cuff connected to an MLT844 Physiological Pressure Transducer (ADInstruments) connected to a PowerLab data acquisition unit (ADInstruments). Dual-energy X-ray absorptiometry was performed

on all rats after 8 and 16 weeks of feeding using a Norland XR46 DXA instrument (Norland Corp., Fort Atkinson, WI, USA). Scans were analysed using the manufacturer's recommended software for use in laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1; Norland Corp. (29).

Oral glucose tolerance tests were performed on rats after overnight deprivation of food. Fructose-supplemented drinking water in H and HS rats was replaced with normal drinking water. Basal blood glucose concentrations were determined in tail vein blood using Medisense Precision Q.I.D. glucometer (Abbott Laboratories, Bedford, MA) and glucose test strips (Freestyle Optium Blood Glucose Test Strips, Abbott Diabetes Care Ltd, Sydney, NSW, Australia). Rats were then given 2 g/kg body weight of glucose as a 40% (w/v) aqueous solution by oral gavage. Following this, blood glucose concentrations were measured at 30, 60, 90 and 120 minutes following glucose administration (29).

Insulin tolerance tests were performed on rats after deprivation of food for four hours. Fructose-supplemented drinking water in H and HS rats was replaced with normal drinking water. Basal blood glucose concentrations were determined in tail vein blood using Medisense Precision Q.I.D. glucometer (Abbott Laboratories, Bedford, MA, USA) and glucose test strips (Freestyle Optium Blood Glucose Test Strips, Abbott Diabetes Care Ltd, Sydney, NSW, Australia). Rats were then given 0.66 IU/kg of insulin (Eli Lilly Australia, West Ryde, Australia) by intraperitoneal injection. Blood glucose concentrations were measured at 30, 60, 90 and 120 minutes after insulin injection (30). If blood glucose concentrations dropped below 1.1 mmol, 4 g/kg body weight of glucose as a 40% (w/v) aqueous solution by oral gavage was immediately administered to prevent hypoglycaemia.

2.6 Measurements after euthanasia

Rats were euthanased by intraperitoneal injection of Lethabarb (pentobarbitone sodium, 100 mg/kg; Virbac, Peakhurst, NSW, Australia). Once euthanasia was induced in rats, heparin was administered (200 IU) into the right femoral vein. The abdomen was then opened and blood (~6 mL) was withdrawn from the abdominal aorta, collected into heparinised tubes, and centrifuged at $5000 \times g$ for 10 minutes. Plasma from each rat was stored at -20°C before analysis. Hearts were removed for use in the isolated Langendorff heart preparation. Hearts were perfused with a modified Krebs-Henseleit bicarbonate buffer (29). Buffer was bubbled with

95% O_2–5% CO_2 and maintained at 35°C. Isovolumetric ventricular function was measured by inserting a latex balloon catheter into the left ventricle connected to a Capto SP844 MLT844 physiological pressure transducer and Chart software on a MacLab system. All left ventricular end-diastolic pressure values were measured during pacing of the heart at 250 beats/minute using an electrical stimulator. End-diastolic pressures were obtained from 0 to 30 mmHg for calculation of diastolic stiffness constant (κ, dimensionless) (29).

After completing the Langendorff heart preparation, hearts were separated into right ventricle and left ventricle with septum for weighing. Livers and abdominal fat pads (retroperitoneal, epididymal and omental) were isolated and weighed. These organ weights were normalised relative to the tibial length at the time of their removal (in mg of tissue/mm of tibial length) (29). Thoracic aortic rings (~4 mm in length) were suspended in an organ bath filled with Tyrode physiological salt solution bubbled with 95% O_2–5% CO_2 maintained at 35°C and stabilised at a resting tension of ~10 mN. Cumulative concentration–response curves (contraction) were obtained for noradrenaline and cumulative concentration–response curves (relaxation) were obtained for acetylcholine and sodium nitroprusside after submaximal (~70%) contraction to noradrenaline (29).

Two rats from each group were exclusively used for histological analysis. Tissues were also collected from two other rats in each group. Approximately 5-7 minutes after euthanasia, heart, liver, ileum and distal colon were collected and fixed in 10% neutral buffered formalin for three days. The samples were then dehydrated and embedded in paraffin wax. Two slides were prepared for each specimen and two random, non-overlapping fields per slide were taken to avoid biased analysis. Approximately 5 μm sections of the samples were cut using a microtome and stained with haematoxylin and eosin for determination of inflammatory cell infiltration and presence of liver fat vacuoles. In addition, liver portions were fixed in Tissue-Tek O.C.T. Compound (ProSciTech, Thuringowa, QLD, Australia) and stored at -20°C. Approximately 10 μm sections of frozen liver in O.C.T. media were cut using a cryostat and stained with Oil Red O to evaluate fat vacuoles. Collagen distribution was defined in the heart with picrosirius red stain. EVOS FL Colour Imaging System (v1.4 (Rev 26059); Advanced Microscopy Group, Bothell, WA, USA) was used to capture images (29). NIH ImageJ software (https://imagej.nih.gov/ij/) was used to quantify

collagen deposition in heart sections, calculate fat vacuole area and to count inflammatory cells in liver sections.

Plasma samples collected during terminal experiments were used to test for enzyme activities and plasma concentrations of biochemical markers. Plasma activities of alanine transaminase (ALT), aspartate transaminase (AST), and plasma concentrations of total cholesterol, triglycerides and non-esterified fatty acids were determined using kits and controls (29).

Immediately after euthanasia and organ removal, two to three faecal pellets were collected from the colon of rats and stored at -80°C in nuclease-free tubes. DNA extraction and diversity profiling were performed by the Centre for Marine Bio-Innovation, University of New South Wales (Kensington, NSW, Australia). For DNA extraction of microbial samples, total microbial community DNA was extracted from faecal samples using the DNeasy Powersoil Kit (Qiagen Australia, Chadstone, VIC, Australia) following the manufacturer's instructions and following the protocol (31). For 16S rRNA gene amplification and sequencing, bacterial communities from faecal samples were investigated by sequencing 16S rRNA gene amplicons. The primers, 341F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCT ACGGGNGGCWGCAG) and 785R (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATC TAATCC) were used to amplify the V3-V4 regions of the 16S rRNA gene. The reaction mixture (50 μL total volume per sample) consisted of Econotaq® PLUS GREEN 2X Master Mix (Astral Scientific, Gymea, NSW, Australia) (25 μL), Ambion® nuclease-free water (17 μL), the primer pair 341F and 785R (1.5 μL of each; 10 μM) and DNA template (5 μL). The PCR program consisted of an initial denaturation at 94°C (2 min), followed by 35 cycles of denaturation at 94°C (30 s), annealing at 55°C (30 s), extension at 72°C (40 s) and a final extension at 72°C (7 min). PCR products were then quantified using gel electrophoresis. Paired-end sequencing (2 x 300 bp) of the resulting 16S rRNA gene amplicons was performed at the Ramaciotti Centre for Genomics, University of New South Wales, on a Illumina MiSeq platform following the MiSeq System User Guide (32). For 16S rRNA gene sequencing analysis, sequence data were initially quality-filtered and trimmed using Trimmomatic version 0.36 truncating reads if the quality dropped below 20 in a sliding window of 4 bp (33). Usearch version 11.0.667 (34) was used for further processing (35) to merge and quality-filter sequencing reads, excluding reads with

< 250 or > 550 nucleotides, in addition to reads with more than one ambiguous base or an expected error of more than 1. Filtered sequences were denoised and clustered into unique sequences (zero-distance operational taxonomic units; zOTUs) using the UNOISE algorithm (36) implemented in USEARCH. zOTUs represent unique bacterial entities and roughly are equivalent to species or strains. Chimeric sequences were removed *de novo* during clustering and subsequently in reference mode using UCHIME (37) with the SILVA database (https://www.arb-silva.de/browser/) (SILVA SSURef 132 NR) as a reference (38). zOTUs were then taxonomically classified (i.e., assigned a likely taxonomic name) by BLASTN (39) against the SILVA database. All non-bacterial OTUs were removed along with non-BLAST aligned and singleton OTUs. Finally, processed sequences were mapped on OTU sequences to calculate the distribution and counts of each OTU in every sample. Only OTUs occurring in more than two samples were considered for further statistical analysis.

2.7 Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Metabolic and physiological results were tested for variance using Bartlett's test and variables that were not normally distributed were transformed using log10 function prior to statistical analyses. Data from the four groups were tested by two-way analysis of variance. When the interactions and/or the main effects were significant, means were compared using the Newman-Keuls multiple comparison *post hoc* test. Where transformations did not result in normality or constant variance, a Kruskal-Wallis non-parametric test was performed. A *p* value of <0.05 was considered as statistically significant. All statistical analyses were performed using Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

For microbiome results, rarefaction curves were generated using the *rarecurve* function in *vegan* (40) and used to determine if a complete representation of the sample's microbiome had been achieved given the sequencing effort. Prior to further analysis, the numbers of sequences were standardised across samples to account for different sequencing depths by randomly subsampling each sample to the lowest number of sequences counts obtained for any given sample (i.e., 14,908 counts). Bacterial alpha-diversities (i.e., zOTU richness and Shannon's diversity) were calculated in R (version 3.5.3) using the *rrarefy* function in the *vegan* package for community ecology analysis (41). A one-way ANOVA test in GraphPad Prism

8.0.2 (San Diego, CA, USA) followed by Tukey's pairwise comparisons test was used to determine the significance between the different groups, a p value of <0.05 was considered as statistically significant.

For multivariate analysis of bacterial communities, OTU tables were imported into PRIMER (42) to compare the community structure (i.e., relative abundance data). Bray-Curtis similarity coefficients were calculated using square-root transformed OTU abundances and the resulting similarity matrix was visualised using non-metric, multi-dimensional scaling (nMDS). Permutational multivariate analysis of variance (PERMANOVA) (43) with 9999 random mutations was used to test the effect of treatment on bacterial communities in rat faecal samples.

3. Results

3.1 Sargassum siliquosum identification

DNA barcoding was used for identification of the *Sargassum* sample collected. A genetic tree was compiled from comparison with a library of DNA barcodes. Phylogenetic trees (Figure 1) show a clustering of similar *Sargassum* species; from this information, this species was identified as *S. siliquosum*. *S. siliquosum* had intact holdfasts and fresh new growth (Figure 2). These samples of *S. siliquosum* contained (in % of dry weight) 57.8% carbohydrate, 1.7% lipid, 4.02% protein, 41.4% dietary fibre including 33.3% as soluble fibre and 7.5% K, 1.9% Ca, 1.2% Na and 0.97% S as the major elements (Tables 1-4).

Table 1. Proximate composition (% of dry weight) of *S. siliquosum*

Lipid	Protein (sum AA)	Ash	Moisture	Carbohydrate*	Dietary fibre		
					Total	Soluble	Insoluble
1.7 ± 0.4	4.02 ± 0.1	27.7 ± 1.3	9.1 ± 0.5	57.8 ± 0.5	41.4 ± 1.7	33.3 ± 2.3	8.2 ± 4.0

* by difference

Values are presented as mean ± SEM, n = 3 for protein and n = 2 for dietary fibre.

Table 2. Elemental composition (% of dry weight) of *S. siliquosum*

C	H	N	S	I
29.00 ± 0.33	4.31 ± 0.09	0.91 ± 0.02	1.18 ± 0.05	0.038 ± 0.002

Values are presented as mean ± SEM, n = 5.

Table 3. Fatty acids and amino acids conntent (% of dry weight) in *S. siliquosum*

Fatty acid	% of dw	Amino acid	% of dw
C14:0	0.12 ± 0	Histidine	0.091 ± 0.001
C15:0	0.07 ± 0	Serine	0.196 ± 0.004
C16:0	0.49 ± 0.02	Arginine	0.209 ± 0.007
C16:1	0.14 ± 0	Glycine	0.214 ± 0.005
C18:0	0.02 ± 0	Aspartic acid	0.466 ± 0.013
C18:1	0.14 ± 0	Glutamic acid	0.607 ± 0.010
C18:2	0.08 ± 0.01	Threonine	0.200 ± 0.004
C18:3	0.12 ± 0.01	Alanine	0.260 ± 0.005
C20:3	0.02 ± 0	Proline	0.177 ± 0.003
C20:4	0.18 ± 0	Lysine	0.222 ± 0.007
C20:5(+C22:0)	0.08 ± 0	Tyrosine	0.120 ± 0.004
		Methionine	0.113 ± 0.006
		Valine	0.241 ± 0.006
		Isoleucine	0.210 ± 0.004
		Leucine	0.345 ± 0.008
		Phenylalanine	0.228 ± 0.005
Total fatty acids	1.47 ± 0.03	Cysteine	0.054 ± 0.002
Sum SFA	0.7 ± 0.02	Tryptophan	0.067 ± 0.003
Sum MUFA	0.28 ± 0	**Sum AA**	**4.02**
Sum PUFA	0.49 ± 0.02	**Sum EAA**	**1.72**

Values are presented as mean ± SEM, n = 3.

Table 4. Metals and metalloids content (mg kg^{-1} of dry weight) in *S. siliquosum*

Element	mg kg^{-1}		
Al	1199	±	179
As	72.9	±	8.1
B	123	±	4
Ba	13.5	±	0.7
Ca	19312	±	925
Cd	0.343	±	0.015
Co	4.90	±	0.29
Cr	3.30	±	0.32
Cu	3.40	±	0.27
Fe	774	±	127
Hg	≤ 0.1		
K	75033	±	2785
Mg	6480	±	265
Mn	104	±	5
Mo	2.01	±	0.75
Na	12226	±	428
Ni	4.12	±	0.21
P	581	±	64
Pb	0.439	±	0.156
S	9681	±	681
Se	4.95	±	0.33
Sr	915	±	49
V	15.5	±	2.6
Zn	18.9	±	2.0

Values are presented as mean ± SEM, n =5.
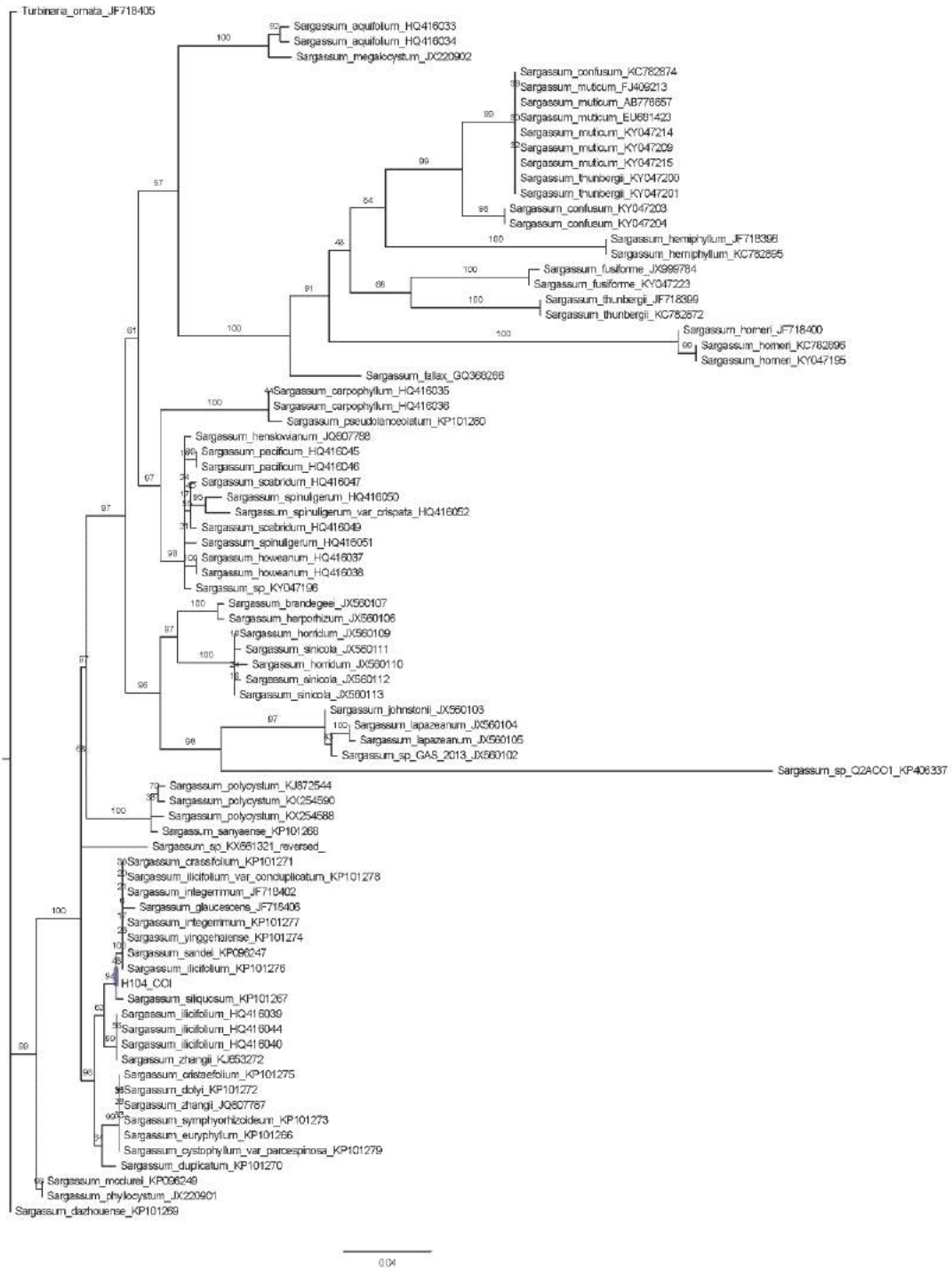
Figure 1. Genetic barcoding of *Sargassum* species: Maximum likelihood tree of cytochrome oxidase I marker sequence data (scale at bottom). Numbers near each node refer to bootstrap support values. Numbers accompanying the species names are GenBank accession numbers for the sequences used in the analysis.

Figure 2. *S. siliquosum* specimen with intact holdfasts and fresh new growth.

3.2 Physiological parameters

After sixteen weeks of feeding, the body weight of H rats was higher than C rats; HS rats were lower than H rats. There was no difference in lean mass across all groups. Fat mass was higher in H rats compared to C rats. Fat mass was similar in CS rats compared to C rats, HS rats had less fat mass than H rats. During weeks 8-16, the food consumption were unchanged in C and CS, and in H and HS (Table 5). CS and HS rats drank more water than their respective controls, C and H. The energy intake of rats was highest in H rats, followed by HS, C and CS. Total abdominal fat was highest in H rats followed by HS, CS and C (Table 5). Plasma triglyceride concentrations were higher in H and HS rats compared to C and CS rats. Plasma total cholesterol and non-esterified fatty acid concentrations were similar in all groups

(Table 5). C rats had lower basal blood glucose concentrations compared to H rats. None of the interventions reduced basal blood glucose concentrations (Table 5). The blood glucose area under the curve was not different between groups (Table 5).

After eight weeks, systolic blood pressure of H diet-fed groups (H and HS) were higher than C diet-fed groups (C and CS) (Table 5). Systolic blood pressure in H rats was higher at 16 weeks than in C rats. HS rats and CS rats had similar systolic blood pressure compared to their respective controls (Table 5). Diastolic stiffness was higher in H rats compared to C rats, HS rats and CS rats showed similar left ventricular diastolic stiffness to their respective controls (Table 5). Left ventricular wet weights with septum and right ventricular wet weights were not different among the groups (Table 5). There was no difference in noradrenaline-induced contraction or sodium nitroprusside-induced relaxation responses of thoracic aorta, while acetylcholine-induced relaxation was improved in HS rats compared to H rats (Figure 3). Left ventricles from H rats showed infiltration of inflammatory cells and collagen deposition whereas these changes were absent in left ventricles from C rats (Figure 4). Collagen deposition in HS and CS rats were similar to their respective controls. Livers from H rats had increased fat vacuole area and infiltration of inflammatory cells compared to livers from C rats (Figure 4). Livers from HS rats had reduced fat vacuoles and inflammatory cells than H rats (Figure 4). Plasma activities of ALT and AST were not different between all groups (Table 5).
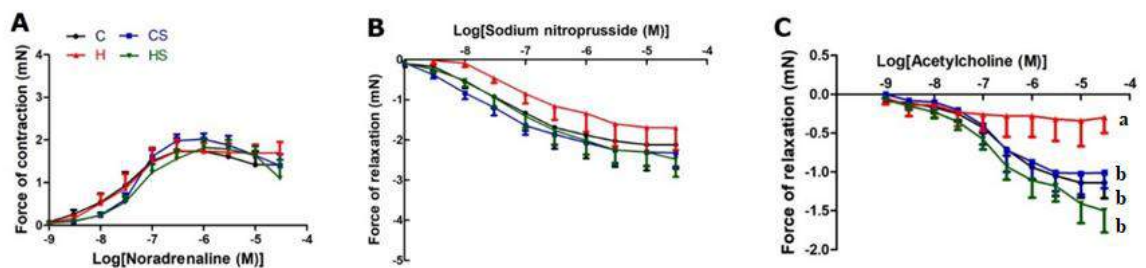
Figure 3. Cumulative concentration-response curves for noradrenaline (A); sodium nitroprusside (B); and acetylcholine (C) in thoracic aorta rings from corn starch diet-fed rats (C), corn starch diet-fed rats supplemented with *S. siliquosum* (CS), high-carbohydrate, high-fat diet-fed rats (H), and high-carbohydrate, high-fat diet-fed rats supplemented with 5% *S. siliquosum* (HS). Means with unlike superscripts (a or b) differ, $p < 0.05$.

Table 5. Responses to *Sargassum siliquosum*

Variable	C	CS	H	HS	P value		
					Diet	**Treatment**	**D x T**
Physiological variables							
0 wk body weight	337 ± 1	338 ± 1	339 ± 1	338 ± 1	0.3807	1	0.3807
8 wk body weight	366 ± 7[b]	369 ± 5[b]	445 ± 10[a]	461 ± 13[a]	<0.0001	0.3873	0.5530
16 wk body weight	388 ± 10[c]	384 ± 10[c]	547 ± 14[a]	490 ±16[b]	<0.0001	0.0472	0.0823
8 wk lean/fat mass proportion	6.2 ± 1.6[a]	3.8 ± 0.5[b]	2.1 ± 0.4[c]	2.0 ± 0.2[c]	0.0001	0.0771	0.1026
16 wk lean/fat mass proportion	3.8 ± 1.0[a]	3.6 ± 0.4[a]	1.5 ± 0.3[b]	1.6 ± 0.2[b]	<0.0001	0.9178	0.7569
16 wk lean mass (g)	292 ± 15	282 ± 6	299 ± 12	281 ± 10	0.7827	0.2035	0.7131
16 wk fat mass (g)	75 ± 15[c]	86 ± 8[c]	248 ± 27[a]	193 ± 19[b]	<0.0001	0.2354	0.0791
16 wk bone mineral content (g)	11.6 ± 0.3[b]	12.2 ± 0.4[b]	16.6 ± 0.9[a]	15.8 ± 0.5[a]	<0.0001	0.8601	0.2227
16 wk bone mineral density (g/cm^2)	0.1703 ± 0.0032	0.1756 ± 0.0034	0.1809 ± 0.0041	0.1811 ± 0.0034	0.0464	0.4841	0.5163
16 wk abdominal circumference (cm)	18.7 ± 0.5[b]	18.5 ± 0.2[b]	21.5 ± 0.2[a]	22.0 ± 0.5[a]	<0.0001	0.7285	0.4200
Visceral adiposity (%)	5.2 ± 0.5[b]	5.3 ± 0.2[b]	9.3 ± 1.1[a]	8.3 ± 0.6[a]	<0.0001	0.4692	0.3773
Body mass index (g/cm^2)	0.61 ± 0.03[b]	0.65 ± 0.01[b]	0.81 ± 0.02[a]	0.75 ± 0.02[a]	<0.0001	0.6248	0.0191
Food intake 0-8 wk (g/day)	43.2 ± 2.2[a]	44.2 ± 1.0[a]	26.6 ± 1.1[b]	26.4 ± 1.0[b]	<0.0001	0.7637	0.6523
Food intake 9-16 wk (g/day)	44.0 ± 1.2[a]	41.1 ± 0.9[a]	23.9 ± 0.9[b]	22.3 ± 0.6[b]	<0.0001	0.0216	0.4902
Water intake 0-8 wk(g/day)	31.8 ± 1.6	31.8 ± 2.0	32.4 ± 1.4	29.1 ± 1.2	0.5693	0.3730	0.3730
Water intake 9-16 wk(g/day)	21.7 ± 1.4[c]	28.5 ± 0.7[b]	28.8 ± 1.3[b]	34.8 ± 1.4[a]	<0.0001	<0.0001	0.7604
Energy intake 0-8 wk (kJ/day)	485 ± [25b]	496 ± 11[b]	607 ± 19[a]	584 ± 20[a]	<0.0001	0.7615	0.3922
Energy intake 9-16 wk (kJ/day)	470 ± 13[b]	457 ± 6[b]	536 ± 15[a]	534 ± 13[a]	<0.0001	0.5471	0.6584
Organ weights							
Liver (mg/mm)	261 ± 11[b]	244 ± 10[b]	380 ± 12[a]	376 ± 14[a]	<0.0001	0.4442	0.6348
Retroperitoneal fat (mg/mm)	210 ± 20[c]	218 ± 13[c]	673 ± 54[a]	495 ± 50[b]	<0.0001	0.0515	0.0341

Epididymal fat (mg/mm)	89 ± 11^b	65 ± 7^b	250 ± 36^a	115 ± 15^b	<0.0001	<0.0001	0.0034
Omental fat (mg/mm)	139 ± 14^b	165 ± 9^b	325 ± 34^a	272 ± 22^a	<0.0001	0.5289	0.0717
Total abdominal fat (mg/mm)	437 ± 42^c	448 ± 25^c	1107 ± 57^a	907 ± 71^b	<0.0001	0.1205	0.0844
Left ventricle + septum (mg/mm)	22.9 ± 1.1	22.8 ± 0.7	25.2 ± 1.1	23.0 ± 0.7	0.1690	0.2047	0.2459
Right ventricle (mg/mm)	4.5 ± 0.7	3.9 ± 0.3	5.3 ± 0.2	5.3 ± 0.2	0.0044	0.4100	0.4100
Kidneys (mg/mm)	52 ± 3^b	49 ± 2^b	58 ± 2^a	60 ± 2^a	0.0011	0.8347	0.3007
Spleen (mg/mm)	14.5 ± 0.7^b	13.6 ± 0.5^b	17.0 ± 0.9^a	16.2 ± 0.8^a	0.0026	0.2851	0.9494
Cardiovascular variables							
8 wk systolic blood pressure (mmHg)	125 ± 4^b	121 ± 2^b	137 ± 3^a	134 ± 3^a	0.0003	0.2678	0.8730
16 wk systolic blood pressure (mmHg)	123 ± 2^b	122 ± 2^b	138 ± 3^a	135 ± 4^a	0.0003	0.5651	0.7732
Diastolic stiffness (κ, dimensionless)	22.1 ± 0.8^b	22.9 ± 0.7^b	30.5 ± 1.2^a	29.4 ± 1.3^a	<0.0001	0.8987	0.4226
Left ventricle collagen area (%)	10 ± 2^b	11 ± 3^b	33 ± 3^a	29 ± 5^a	0.0003	0.6733	0.4866
Metabolic variables							
Total cholesterol (mmol/L)	1.56 ± 0.08	1.68 ± 0.06	1.57 ± 0.10	1.86 ± 0.19	0.5300	0.1802	0.5740
Triglycerides (mmol/L)	0.43 ± 0.02^b	0.42 ± 0.04^b	1.88 ± 0.31^a	1.54 ± 0.28^a	<0.0001	0.3986	0.4258
Nonesterified fatty acids (mmol/L)	0.38 ± 0.06	0.36 ± 0.03	0.40 ± 0.03	0.38 ± 0.04	0.6421	0.6421	1
ALT (U/L)	34 ± 4	53 ± 6	38 ± 2	48 ± 6	0.9371	0.0276	0.4790
AST (U/L)	116 ± 2	150 ± 19	120 ± 12	142 ± 20	0.9219	0.1762	0.7688
Liver inflammatory cells (cells/200μm^2)	6 ± 1^b	7 ± 1^b	25 ± 2^a	26 ± 3^a	<0.0001	0.6195	1
Liver fat vacuole area (μm^2)	13.1 ± 1.7^c	15.6 ± 2.4^c	88.6 ± 3.4^a	55.2 ± 2.9^b	<0.0001	<0.0001	<0.0001
Oral glucose tolerance							
0 wk basal glucose (mmol/L)	2.6 ± 0.1	2.6 ± 0.1	2.6 ± 0.2	2.7 ± 0.1	0.6968	0.6968	0.6968
0 wk AUC (mmol/L/120 minutes)	632 ± 30	594 ± 20	606 ± 19	552 ± 12	0.1102	0.0334	0.7016
8 wk basal glucose (mmol/L)	2.9 ± 0.2^b	2.6 ± 0.1^b	3.3 ± 0.1^a	3.5 ± 0.1^a	<0.0001	0.6968	0.0580
8 wk 120 min glucose (mmol/L)	3.5 ± 0.2^b	3.7 ± 0.1^b	5.0 ± 0.1^a	5.2 ± 0.2^a	<0.0001	0.2692	1

	C	CS	H	HS	Diet	S. siliquosum	Interaction
8 wk AUC (mmol/L/120 min)	530 ± 15[b]	537 ± 9[b]	657 ± 22[a]	682 ± 15[a]	<0.0001	0.3103	0.5660
16 wk basal glucose (mmol/L)	2.8 ± 0.2	3.0 ± 0.2	3.3 ± 0.2	3.4 ± 0.1	0.5467	0.8404	0.9465
16 wk 120 min glucose (mmol/L)	3.9 ± 0.2[b]	3.7 ± 0.1[b]	4.8 ± 0.3[a]	4.5 ± 0.1[a]	<0.0001	0.1276	0.7564
16 wk AUC (mmol/L/120 min)	501 ± 21[b]	523 ± 14[b]	617 ± 25[a]	604 ± 9[a]	<0.0001	0.7851	0.2929
Insulin tolerance							
8 wk ITT 120 min glucose (mmol/L)	2.9 ± 0.4[b]	2.7 ± 0.2[b]	4.5 ± 0.3[a]	4.4 ± 0.2[a]	<0.0001	0.5762	0.8519
8 wk ITT AUC (mmol/L/120 min)	247 ± 58[b]	234 ± 32[b]	408 ± 21[a]	390 ± 18[a]	<0.0001	0.6494	0.9415
16 wk ITT 120 min glucose (mmol/L)	2.7 ± 0.3[b]	3.3 ± 0.2[b]	4.5 ± 0.4[a]	4.3 ± 0.2[a]	<0.0001	0.4570	0.1419
16 wk ITT AUC (mmol/L/120 min)	208 ± 37[b]	168 ± 27[b]	404 ± 54[a]	420 ± 28[a]	<0.0001	0.7397	0.4400

Values are presented as mean ± SEM, n = 10-12. Means in a row with unlike superscripts (a, b or c) differ, $p < 0.05$. C, corn starch diet-fed rats; CS, corn starch diet-fed rats supplemented with 5% *S. siliquosum*; H, high-carbohydrate, high-fat diet-fed rats; HS, high-carbohydrate, high-fat diet-fed rats supplemented with 5% *S. siliquosum.* ALT, alanine transaminase; AST, aspartate transaminase; AUC, area under the curve; ITT, insulin tolerance test.
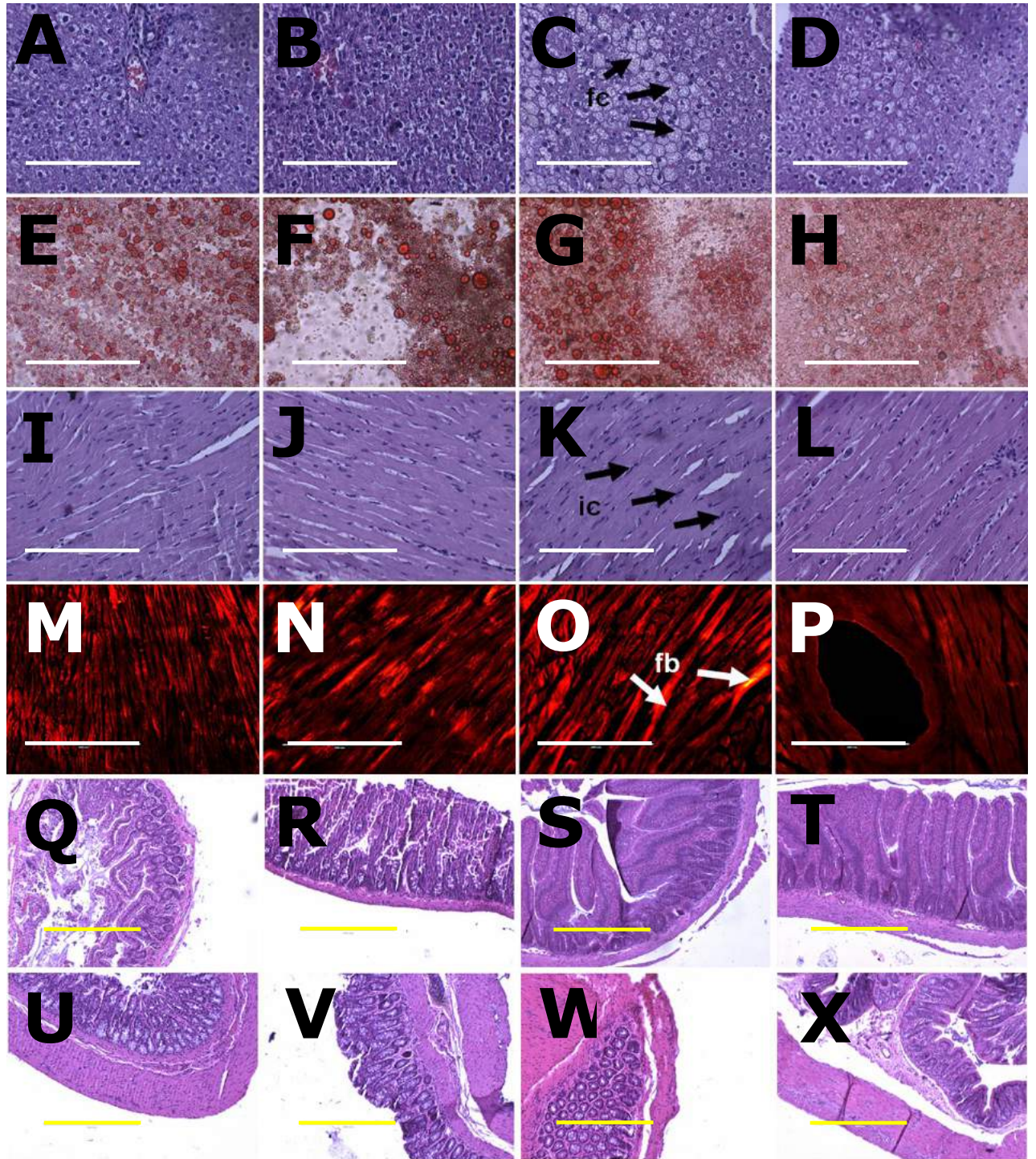
Figure 4. Liver fat using haematoxylin and eosin stain (A-D) and oil red O stain (E-H); heart inflammation (I-L) using haematoxylin and eosin stain; heart fibrosis (M-P) using picrosirius red stain; ileum (Q-T) and colon (U-X) structure using haematoxylin and eosin stain. Each row is in the following order: corn starch diet-fed rats (C), corn starch diet-fed rats supplemented with 5% *S. siliquosum* (CS), high-carbohydrate, high-fat diet-fed rats (H) and high-carbohydrate, high-fat diet-fed rats supplemented

with 5% *S. siliquosum* (HS). Fat cells=fc; inflammatory cells=ic; fibrosis=fb. Scale bar is 200 μm for A-P (20x) and 100 μm for Q-X (10x).

3.3 Gut structure and microbiome

The ileum and colon did not show any histological abnormalities demonstrated by normal crypt depth, villi length, goblet cells and lack of inflammatory cell infiltration in the experimental groups (Figure 4).

The gut microbiome was defined as the collective genomes of the microbes in the rat's colon. After quality filtering there were a total of 799,215 sequences and these were clustered into 1307 zOTUs. The calculated rarefaction curves based on rarefied and unrarefied data as well as Good's coverage of 99.69 ± 0.12% revealed that the majority of the bacterial community was recovered by the surveying effort. There was no statistical support for differences in Shannon's diversity or richness between the four groups. An overall effect of treatment was observed on the bacterial community structure for the faecal samples (Figure 5). H rats showed increases in relative abundance of *Firmicutes* and decreases in relative abundance of *Bacteroidetes* compared to C rats indicating an effect of basal feed on the bacterial community structure. The addition of *S. siliquosum* (CS and HS) did not change the bacterial communities.
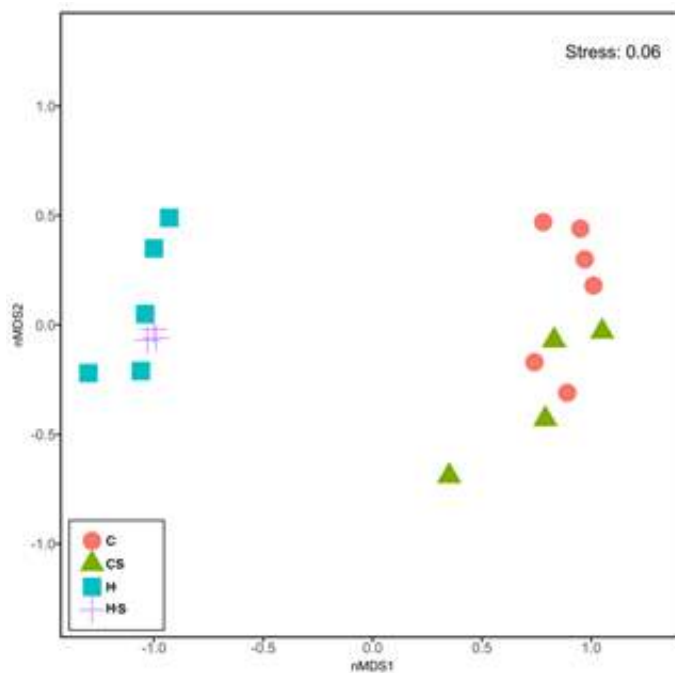

Figure 5. MDS plot of bacterial communities structure of faecal samples from different diets. Corn starch diet-fed rats (C), corn starch diet-rats supplemented with

5% *S. siliquosum* (CS), high-carbohydrate, high-fat diet-fed rats (H) and high-carbohydrate, high-fat diet-fed rats supplemented with 5% *S. siliquosum* (HS).

4. *Discussion*

Seaweeds contain polysaccharides, peptides, pigments, minerals and omega-3 fatty acids which may reduce the characteristic signs of obesity, diabetes, hypertension, fatty liver and inflammation in metabolic syndrome (44). There have been several studies showing anti-obesity actions from brown seaweeds (45). *Sargassum* species contain bioactive compounds that have demonstrated therapeutic potential and could be introduced for the preparation of novel functional ingredients in pharmaceuticals for the treatment and or prevention of several disorders (3). For example, *S. polycystum* suppressed weight gain in rats fed with high-fat diet and reduced the plasma concentrations of cholesterol and triglycerides (46) and *Undaria pinnatifida* decreased body weight gain and energy consumption and reduced serum levels of glucose and insulin in diet-induced obese mice (47).

This study showed that 5% *S. siliquosum* supplementation decreased body weight and body fat, decreased retroperitoneal fat and liver fat but had no effect on systolic blood pressure, liver enzyme activities, lipid profile or glucose and insulin metabolism in rats with diet-induced metabolic syndrome. *Sargassum* species contain polysaccharides, mainly (in decreasing content) alginates, fucoidans and laminaran (Figure 6); these are likely to be the major constituents of the high fibre content of *S. siliquosum* (15). Fucoidans and alginates obtained from various *Sargassum* species have a wide range of molecular weights, from 8 to 627 kDa (15). The prebiotic effect of soluble fibre is due to the polysaccharides being resistant to hydrolysis in the upper gastrointestinal tract (48). Additionally, the biological responses could be due to the anti-oxidant (49) effects from the high phenolic content in brown seaweeds (50), as well as the xanthophyll, fucoxanthin (51). There is limited literature on the health benefits of *Sargassum*.

Alginates are mainly linear polymers consisting of β-D-mannuronic and α-L-guluronic acids with different mannuronic/guluronic acid ratios and linear arrangements (15) and comprise up to 40% of the dry weight of brown seaweeds (52). As alginates can absorb water and form viscous gum, they have been used as thickeners, stabilisers and gelling agents in the food and pharmaceutical industries (33). The formation of alginate gels in the small intestine is the most likely mechanism

for alginate-related body weight loss following slowed nutrient absorption (53). In limited animal studies, *Sargassum* species have shown further activities that could lead to loss of body weight. *S. siliquosum* inhibited low-density lipoprotein oxidation, angiotensin converting enzyme, α-amylase and α-glucosidase which reduces the risk of developing cardiovascular diseases (23). In a high-fat rat model, 10% *S. polycystum* supplementation reduced body weight and decreased total cholesterol and triglyceride plasma concentrations (46).
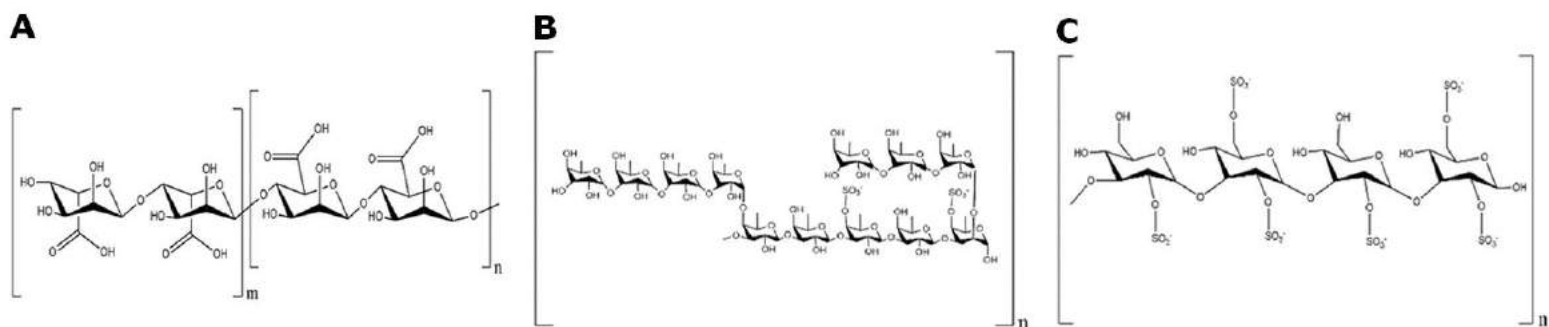
Figure 6. Alginate (A), fucoidan (B) and laminaran (C) chemical structures.

Alginates have diverse applications including as biopolymers for nanoparticles because they are readily available, low cost, non-toxic, biodegradable and versatile, and therefore allow targeted drug delivery (54). Nanoparticles have been used to delivery nutraceuticals which provide health benefits and decrease risk of several chronic diseases (55). Nanoparticles can overcome negative characteristics of natural products such as low bioavailability of curcumin. In the same rat model as the current study, poly lactic–co–glycolic acid nanoparticles increased the bioavailability of curcumin by ~20-fold demonstrating that a low dose of curcumin encapsulated within nanoparticles to have a similar effect of lowering systolic blood pressure as a high dose of unformulated curcumin (56); alginate nanoparticles could show similar effects. Fucoidans have also been used in nanomedicine because of their versatility with regard to chemical modifications, and relatively low production costs (57).

Another use of alginates is as a bioplastic. Alginate extracted from *S. siliquosum* to produce biodegradable plastic was used as an alternative to conventional plastic to address pollution and environmental issues (58). *Sargassum* may also be useful as biological charcoal (biochar) and can been used to improve soil productivity and act as a fertiliser because of their high concentrations of N, P and K (59). Additionally, the biosorption capacity of these seaweeds for heavy metals (e.g.,

selective metal binding) resides mainly in alginates that occur as a gel in the algal thallus (60). *S. oligocystum* been used as an effective biosorbent for the treatment of heavy metals in wastewater (61). Further, an adequate amount of K, N, growth promoting hormones, micronutrients and humic acids present in seaweeds make it an excellent fertiliser (62). Unlike chemical fertilisers, fertilisers derived from seaweeds such as *Fucus*, *Laminaria*, *Ascophyllum* and *Sargassum* are biodegradable, non-toxic, non-polluting and non-hazardous to humans, animals and birds (63). Furthermore, seaweeds are currently harvested primarily for high-value natural products and food but they have the potential to be used as fertilisers due to the high yields (64) being 6.5 times more productive than sugarcane (65). Thus, the commercial value of *Sargassum* may be increased if multiple revenue streams are fully explored and developed. In particular, the polysaccharides (alginate, fucoidan and laminaran) and pigments (chlorophyll a and c, *β*-carotene and fucoxanthin) can be used for health benefits as a functional food; and the polysaccharide portion extracted for drug delivery, fertilisers, targeted biosorption bioremediation and biofuel production (66, 67).

In vitro fermentation of a polysaccharide from *S. thunbergii* increased concentrations of total short chain fatty acids, acetic, propionic, *n*-butyric and *n*-valeric acids (68). *Sargassum* may act as a prebiotic which provides fermentable substrates to enable selective gut commensal metabolism in obesity (48).

5. Conclusions

These results with *S. siliquosum* provide evidence that brown seaweeds contain bioactive compounds such as alginates, fucoidans and fucoxanthin that attenuate some symptoms of diet-induced metabolic syndrome in rats. The mechanism of action is likely through a combination of prebiotic effects and systemic anti-inflammatory actions in the heart and liver. Further mechanistic studies would be valuable to determine the *in vivo* actions of the alginate and fucoidan components that may be responsible for the health benefits.

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CHAPTER 5. GREEN SEAWEEDS IN DIET-INDUCED METABOLIC SYNDROME

The previous chapters have concentrated on seaweeds grown for commercial reasons that may also have health benefits. This chapter shifts the emphasis to a seaweed, *Caulerpa lentillifera* known as "sea grapes", widely consumed in the Philippines, Indonesia and Vietnam as a low-energy but nutrient-rich food. This seaweed contains a range of compounds widely considered as important components of healthy foods, including proteins, minerals, dietary fibre, vitamins, and saturated and unsaturated fatty acids. This chapter investigated the potential health benefits of this green seaweed to reverse diet-induced metabolic syndrome in rats. The main polysaccharides in *C. lentillifera* are sulphated polysaccharides. Although *C. lentillifiera* is a commerically available product in Vietnam, its effects on metabolic syndrome have not been reported. My hypothesis is that *C. lentillifera* will reverse the cardiovascular, liver and metabolic changes induced in rats by a high-carbohydrate, high-fat diet. This chapter builds on the previous chapters where other sulphated polysaccharides, including κ- and ι-carrageenans from the red seaweeds, *K. alvarezii* and *S. filiforme* and non-sulphated polysaccharides such as alginates and laminarans from the brown seaweed *S. siliquosum* prevented or attenuated the signs of metabolic syndrome. Changes in the gut microbiome may occur following ingestion of seaweeds, so this important area of research will also be investigated to determine possible mechanisms of action for the observed physiological responses to *C. lentillifera*.

Caulerpa lentillifera (sea grapes) improves cardiovascular and metabolic health of rats with diet-induced metabolic syndrome

Ryan du Preez [1,2], Lindsay Brown [1,2], Marwan Majzoub [3], Torsten Thomas [3], Sunil K Panchal [1,*]

[1]Functional Foods Research Group, University of Southern Queensland, Toowoomba, QLD 4350, Australia

[2]School of Health and Wellbeing, University of Southern Queensland, Toowoomba, QLD 4350, Australia

[3]Centre for Marine Bio-Innovation, University of New South Wales, Sydney, NSW 2052, Australia

*Correspondence: Sunil.Panchal@usq.edu.au; Tel.: +61 7 4631 1012

Abstract

Caulerpa lentillifera (sea grapes) are widely consumed in south-east Asia as low-energy foods with high contents of vitamins and minerals. Dried *C. lentillifera* contains 44% carbohydrates, 14% lipids, 7% protein, 17.5% total dietary fibre including 16.6% insoluble fibre. This study investigated dried sea grapes commercially produced in Vietnam for their effects in reversing diet-induced metabolic syndrome. Male Wistar rats (n = 48) were randomly allocated to four groups in a 16-week protocol. Two groups were fed either corn starch (C) or high-carbohydrate, high-fat (H) diets for the full 16 weeks. The other two groups received C and H diets for eight weeks and then received *C. lentillifera* added to these diets for the final eight weeks (CCL and HCL, respectively). The H diet had increased fructose and sucrose, together with increased saturated and *trans* fats. H rats developed obesity, hypertension, dyslipidaemia, fatty liver disease and increased left ventricular collagen deposition. *C. lentillifera* supplementation in HCL decreased body weight (C, 411±14; CCL, 389±6; H, 573±10; HCL, 468±10 g), systolic blood pressure (C, 119±3; CCL, 117±4; H, 135±3; HCL, 118±3 mmHg), plasma total cholesterol (C, 1.59±0.06; CCL, 1.54±0.05; H, 1.73±0.09; HCL, 1.56±0.06 mmol/L), non-esterified fatty acids (C, 0.68±0.12; CCL, 0.47±0.04; H, 2.71±0.29; HCL, 1.61±0.49 mmol/L) concentrations and visceral adiposity (C, 5.1±0.2; CCL, 4.3±0.2; H, 9.8±0.8; HCL, 7.7±0.7%). Therefore, *C. lentillifera* attenuated cardiovascular and metabolic symptoms of metabolic syndrome in rats.

Keywords: *Caulerpa lentillifera*; sea grapes; green seaweed; gut microbiome; metabolic syndrome

1. Introduction

Seaweeds are an important source of macronutrients and micronutrients, especially in East and South-East Asia as a major part of the traditional diet (1, 2). The *Caulerpa* genus consists of about 75 species of tropical to sub-tropical siphonous green seaweed (3). *Caulerpa lentillifera* known as "sea grapes" (4), consumed in the Philippines, Indonesia and Vietnam, contains proteins, minerals, dietary fibre, vitamins, and saturated and unsaturated fatty acids (5). Foods containing these components have been investigated to prevent or reverse metabolic syndrome (6), defined as a clustering of cardiometabolic risk factors such as obesity, dyslipidaemia, hypertension and glucose intolerance (7).

Decreases in these cardiometabolic risk factors have been reported for *C. lentillifera*, especially improved glucose metabolism and reduced inflammation which are key symptoms of metabolic syndrome. As examples, *C. lentillifera* decreased body weight, reduced plasma trigylcerides and increased HDL-C concentrations in high-cholesterol, high-fat diet-fed rats (8). *C. lentillifera* extract at 250 mg/kg and 500 mg/kg body weight for six weeks decreased fasting blood glucose concentrations in oral glucose tolerance test and intraperitoneal insulin tolerance test in C57BL/KsJ-db/db mice. Furthermore, dosage with 500 mg/kg body weight decreased glycogen content by 54.8% (9). Polysaccharide fractions purified from *C. lentillifera* enhanced immunostimulatory activity which increased the proliferation of macrophages, increased phagocytosis, nitric oxide production and acid phosphatase activity in macrophages (10). Reduced inflammation may be due to enhanced immunostimulatory activity through increased production of short-chain fatty acids and gut bacteria diversity and composition in immunosupressed BALB/c mice by interacting with immune cells and enterocytes in regulating and maintaining the normal function of the innate and adaptive immune system (11). Further, these immune responses were correlated with improved growth in *Lactobacillus*, *Coriobacteriaceae*, *Ruminococcaceae*, *Clostridium_XVIII* and *Helicobacter* and suppressed populations of *Bacteroides*, *Barnesiella* and *Lachnospiraceae* (11).

The aim of this study was to determine whether diet-induced changes in cardiovascular, liver and metabolic responses can be attenuated by chronic dietary intervention with dried *C. lentillifera*. We chose this *Caulerpa* species as a controlled aquaculture study reported an increased yield of 2 kg fresh weight/week compared to *C. racemosa* which yielded <0.5 kg fresh weight/week (4, 12). We sourced *C.*

lentillifera from Vietnam because it is produced there as a commercial product. A validated diet-induced rat model of metabolic syndrome that closely mimics the symptoms of human metabolic syndrome (13) was used for this study. We measured systolic blood pressure, left ventricular diastolic stiffness, inflammatory cells and collagen deposition as cardiovascular responses; plasma liver enzyme activities, liver inflammatory cells and fat vacuoles as liver responses; and body weight and composition, total cholesterol and triglyceride concentrations, and glucose and insulin tolerance as metabolic responses. We hypothesised that 5% *C. lentillifera* supplementation for the last eight weeks of the protocol will reverse the changes in these parameters induced by the high-carbohydrate, high-fat diet.

2. Materials and Methods

2.1 C. lentillifera source and elemental composition analysis

C. lentillifera was purchased from Viet Delta Corporation, Ho Chi Minh City, Vietnam. 5 kg of dried *C. lentillifera* was transported to the University of Southern Queensland, Toowoomba, QLD, Australia, in vacuum-sealed bags containing silica desiccant. Soluble and insoluble dietary fibre was analysed by a commercial laboratory (Symbio Laboratories, Brisbane, QLD, Australia). For elemental composition analysis, about 0.1 g of *C. lentillifera* biomass was placed in a digestion vessel. 3.5 mL of double-distilled HNO_3 and 0.5 mL of superpure H_2O_2 was added. The mixture was left in a fumehood for about 1 h before it was loaded into a Berghof Speedwave microwave digestion system for sample digestion. Then the solution was quantitatively transferred into a 50 mL volumetric flask and filled up to the mark using Milli-Q water. This solution was analysed for Al, Ca, Mg, K, Na, P and S by an Agilent 5100 ICP-OES and the other elements were analysed by a Varian 820 ICP-MS (Advanced Analytical Centre, James Cook University, Townsville, QLD, Australia).

2.2 Rats and diets

All experimental protocols were approved by the Animal Ethics Committee of the University of Southern Queensland (approval number 16REA014) under the guidelines of the National Health and Medical Research Council of Australia. Male Wistar rats (8-9 weeks old; 338 ± 1 g, n = 48) were obtained from the Animal Resource Centre, Murdoch, WA, Australia. Rats were individually housed in a temperature-controlled (21±2ºC), 12-hour light/dark conditions with free access to food and water. Rats were randomly allocated to four groups, each of 12 rats. Two groups were fed

either corn starch or high-carbohydrate, high-fat diets (C and H, respectively) (13) for the full 16 weeks. The other two groups received C and H diets for eight weeks and then received 5% dried *C. lentillifera* mixed in the food for the final eight weeks (CCL and HCL, respectively).

2.3 Measurements before terminal anaesthesia

A mediquip anaesthesia machine was used to induce light sedation to immobilise the rat. Rats were pre-oxygenated with 2 L/min for three minutes prior to 5% isoflurane induction vaporised in 1.5 L/min. Rats were placed in a nose mask and maintained with 1.5% isoflurane in 1.5 L/min O_2 for the duration of each procedure. Systolic blood pressure measurements were performed on all rats after 8 and 16 weeks of feeding using an MLT1010 Piezo-Electric Pulse Transducer (ADInstruments, Bella Vista, NSW, Australia) and an inflatable tail-cuff connected to an MLT844 Physiological Pressure Transducer (ADInstruments) connected to a PowerLab data acquisition unit (ADInstruments). Dual-energy X-ray absorptiometry was performed after 8 and 16 weeks of feeding using a Norland XR46 DXA instrument (Norland Corp., Fort Atkinson, WI, USA). Scans were analysed using the manufacturer's recommended software for use in laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1; Norland Corp. (13).

Oral glucose tolerance tests were performed on rats after overnight deprivation of food. Fructose-supplemented drinking water in H and HCL rats was replaced with normal drinking water. Basal blood glucose concentrations were determined in tail vein blood using Medisense Precision Q.I.D. glucometer (Abbott Laboratories, Bedford, MA, USA) and glucose test strips (Freestyle Optium Blood Glucose Test Strips, Abbott Diabetes Care Ltd, Sydney, NSW, Australia). Rats were then given 2 g/kg body weight of glucose as a 40% (w/v) aqueous solution by oral gavage. Following this, blood glucose concentrations were measured at 30, 60, 90 and 120 minutes following glucose administration (14). Insulin tolerance tests were performed on rats after deprivation of food for four hours. Fructose-supplemented drinking water in H and HCL rats was replaced with normal drinking water. Basal blood glucose concentrations were determined in tail vein blood using Medisense Precision Q.I.D. glucometer (Abbott Laboratories, Bedford, MA, USA) and glucose test strips (Freestyle Optium Blood Glucose Test Strips, Abbott Diabetes Care Ltd, Sydney, NSW, Australia). Rats were then given 0.66 IU/kg of insulin (Eli Lilly Australia, West

Ryde, Australia) by intraperitoneal injection. Following this, blood glucose concentrations were measured at 30, 60, 90 and 120 minutes following insulin administration (15). If blood glucose concentrations dropped below 1.1 mmol, 4 g/kg body weight of glucose as a 40% (w/v) aqueous solution was immediately administered by oral gavage to prevent hypoglycaemia.

Indirect calorimetry was used to measure oxygen consumption and carbon dioxide production using a 4-chamber Oxymax system (Columbus Instruments, Columbus, OH, USA) with one rat per chamber. Rats had free access to food and water during the measurement. Oxygen consumption (V_{O2}) and carbon dioxide production (V_{CO2}) were measured individually from each chamber. The respiratory exchange ratio (RER = V_{CO2}/V_{O2}) was calculated by Oxymax software (v. 4.86). The oxidation of carbohydrates produces an RER of 1.00, whereas fatty acid oxidation results in an RER of about 0.70. Energy expenditure (heat) was calculated by assessment of the exchange of oxygen for carbon dioxide that occurs during the metabolic processing of food (16).

2.4 Measurements after terminal anaesthesia

Terminal anaesthesia was induced in rats by intraperitoneal injection of Lethabarb (pentobarbitone sodium, 100 mg/kg; Virbac, Peakhurst, NSW, Australia). Once terminal anaesthesia was induced in rats, heparin (200 IU) was administered into the right femoral vein. The abdomen was then opened and blood (~6 mL) was withdrawn from the abdominal aorta, collected into heparinised tubes, and centrifuged at $5000 \times g$ for 10 minutes. Plasma from each rat was stored at -20°C before analysis. Hearts were removed for use in the isolated Langendorff heart preparation. Hearts were perfused with a modified Krebs-Henseleit bicarbonate buffer (13). Buffer was bubbled with 95% O_2–5% CO_2 and maintained at 35°C. Isovolumetric ventricular function was measured by inserting a latex balloon catheter into the left ventricle connected to a Capto SP844 MLT844 physiological pressure transducer and Chart software on a MacLab system. Left ventricular end-diastolic pressure values were measured during pacing of the heart at 250 beats/minute using an electrical stimulator. End-diastolic pressures were obtained from 0 to 30 mmHg for calculation of diastolic stiffness constant (κ, dimensionless) (13).

After completing the Langendorff heart preparation, hearts were separated into right ventricle and left ventricle with septum for weighing. Livers and abdominal fat

pads (retroperitoneal, epididymal and omental) were isolated and weighed. These organ weights were normalised relative to the tibial length at the time of their removal (in mg of tissue/mm of tibial length) (13).

Two rats from each group were exclusively used for histological analysis. Tissues were also collected from two other rats in each group. Approximately 5-7 minutes after euthanasia, heart, liver and intestinal tissues were collected and fixed in 10% neutral buffered formalin for three days. The samples were then dehydrated and embedded in paraffin wax. Two slides were prepared for each heart and liver specimen and two random, non-overlapping fields per slide were taken to avoid biased analysis. Thin sections (5 μm) of the samples were cut and stained with haematoxylin and eosin for determination of inflammatory cell infiltration and presence of liver fat vacuoles. In addition, liver portions were fixed in Tissue-Tek O.C.T. Compound (ProSciTech, Thuringowa, QLD, Australia) and stored at -20°C. Approximately 10 μm sections of frozen liver in O.C.T. media were cut using a cryostat and stained with Oil Red O to evaluate fat vacuoles. Collagen distribution was defined in the heart with picrosirius red stain. EVOS FL Colour Imaging System (v1.4 (Rev 26059); Advanced Microscopy Group, Bothell, WA, USA) was used to capture images (13). NIH ImageJ software (https://imagej.nih.gov/ij/) was used to quantify collagen deposition in heart sections, calculate mean fat vacuole area and to count inflammatory cells in liver sections.

Plasma samples collected during terminal experiments were used to test for enzyme activities and plasma concentrations of biochemical markers. Plasma activities of alanine transaminase (ALT) and aspartate transaminase (AST), and plasma concentrations of total cholesterol, triglycerides and non-esterified fatty acids were determined using appropriate kits and controls (13). Plasma HDL- and LDL-cholesterol concentrations were analysed using commercially available ELISA kits (Abcam, Australia).

Immediately after euthanasia and organ removal, two to three faecal pellets were collected from the colon of rats and stored at -80°C in nuclease-free tubes. DNA extraction and diversity profiling were performed by the Centre for Marine Bio-Innovation, University of New South Wales (Kensington, NSW, Australia). For DNA extraction of microbial samples, total microbial community DNA was extracted from faecal samples using the DNeasy Powersoil Kit (Qiagen Australia, Chadstone, VIC, Australia) following the manufacturer's instructions and following the protocol (17).

For 16S rRNA gene amplification and sequencing, bacterial communities from faecal samples were investigated by sequencing 16S rRNA gene amplicons. The primers, 341F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCT ACGGGNGGCWGCAG) and 785R (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATC TAATCC) were used to amplify the V3-V4 regions of the 16S rRNA gene. The reaction mixture (50 μL total volume per sample) consisted of Econotaq® PLUS GREEN 2X Master Mix (Astral Scientific, Gymea, NSW, Australia) (25 μL), Ambion® nuclease-free water (17 μL), the primer pair 341F and 785R (1.5 μL of each; 10 μM) and DNA template (5 μL). The PCR program consisted of an initial denaturation at 94°C (2 min), followed by 35 cycles of denaturation at 94°C (30 s), annealing at 55°C (30 s), extension at 72°C (40 s) and a final extension at 72°C (7 min). PCR products were then quantified using gel electrophoresis. Paired-end sequencing (2 x 300 bp) of the resulting 16S rRNA gene amplicons was performed at the Ramaciotti Centre for Genomics, University of New South Wales on a Illumina MiSeq platform following the MiSeq System User Guide (18). For 16S rRNA gene sequencing analysis, sequence data were initially quality-filtered and trimmed using Trimmomatic version 0.36 truncating reads if the quality dropped below 20 in a sliding window of 4 bp (19). USEARCH version 11.0.667 (20) was used for further processing (21) to merge and quality-filter sequencing reads, excluding reads with < 250 or > 550 nucleotides, in addition to reads with more than one ambiguous base or an expected error of more than 1. Filtered sequences were denoised and clustered into unique sequences (zero-distance operational taxonomic units; zOTUs) using the UNOISE algorithm (22) implemented in USEARCH. zOTUs represent unique bacterial entities and roughly are equivalent to species or strains. Chimeric sequences were removed *de novo* during clustering and subsequently in reference mode using UCHIME (23) with the SILVA database (https://www.arb-silva.de/browser/) (SILVA SSURef 132 NR) as a reference (24). zOTUs were then taxonomically classified (i.e. assigned a likely taxonomic name) by BLASTN (25) against the SILVA database. All non-bacterial OTUs were removed along with non-BLAST aligned and singleton OTUs. Finally, processed sequences were mapped on OTU sequences to calculate the distribution and counts of each OTU in every sample. Only OTUs occurring in more than two samples were considered for further statistical analysis.

2.5 Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Metabolic and physiological results were tested for variance using Bartlett's test and variables that were not normally distributed were transformed using log10 function prior to statistical analyses. Data from the four groups were tested by two-way analysis of variance. When the interactions and/or the main effects were significant, means were compared using the Newman-Keuls multiple comparison *post hoc* test. Where transformations did not result in normality or constant variance, a Kruskal-Wallis non-parametric test was performed. A *p* value of <0.05 was considered as statistically significant. All statistical analyses were performed using Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

For microbiome results, rarefaction curves were generated using the *rarecurve* function in *vegan* (26) and used to determine if a complete representation of the sample's microbiome had been achieved given the sequencing effort. Prior to further analysis, the numbers of sequences were standardised across samples to account for different sequencing depths by randomly subsampling each sample to the lowest number of sequences counts obtained for any given sample (i.e., 14,908 counts). Bacterial alpha-diversities (i.e., zOTU richness and Shannon's diversity) were calculated in R (version 3.5.3) using the *rrarefy* function in the *vegan* package for community ecology analysis (26). A one-way ANOVA test in GraphPad Prism 8.0.2 (San Diego, CA, USA) followed by Tukey's pairwise comparisons test was used to determine the significance between the different groups, a *p* value of <0.05 was considered as statistically significant.

For multivariate analysis of bacterial communities, OTU tables were imported into PRIMER (27) to compare the community structure (i.e., relative abundance data). Bray-Curtis similarity coefficients were calculated using square-root transformed OTU abundances and the resulting similarity matrix was visualised using non-metric, multi-dimensional scaling (nMDS). Permutational multivariate analysis of variance (PERMANOVA) (28) with 9999 random mutations was used to test the effect of treatment on bacterial communities in rat faecal samples. 'Treatment' was set with corn-starch diet (C), corn-starch diet supplemented with *C. lentillifera* (CCL), high-carbohydrate, high-fat diet (H) and high-carbohydrate, high-fat diet supplemented with *C. lentillifera* (HCL) as fixed factors.

3. Results

3.1 *C. lentillifera* composition

The *C. lentillifera* powder contained (in % dry weight) 16% moisture, 44% carbohydrates, 14% lipids, 7% protein and 17.5% total dietary fibre including 16.6% insoluble fibre. The predominant compounds in the *C. lentillifera* biomass were 13.1% Na, 1.1% Mg, 0.81% Ca and 0.67% S (Table 1).

Table 1. Elemental composition of *C. lentillifera* biomass

Element	Concentration (mg/kg)
Aluminium (Al)	744 ± 8
Arsenic (As)	≤1
Boron (B)	21.7 ± 0.5
Barium (Ba)	4.75 ± 0.19
Calcium (Ca)	8137 ± 73
Cadmium (Cd)	1.14 ± 0.03
Cobalt (Co)	1.35 ± 0.05
Chromium (Cr)	3.3 ± 0.5
Copper (Cu)	2.74 ± 0.3
Iron (Fe)	595 ± 5
Mercury (Hg)	≤1
Potassium (K)	1066 ± 546
Magnesium (Mg)	10663 ± 52
Manganese (Mn)	425 ± 14
Sodium (Na)	130794 ± 763
Molybdenum (Mo)	1.32 ± 0.14
Nickel (Ni)	1.88 ± 0.19
Phosphate (P)	1073 ± 17
Lead (Pb)	2.22 ± 0.08
Sulphur (S)	6733 ± 72
Selenium (Se)	≤1
Strontium (Sr)	104 ± 3
Vanadium (V)	2.46 ± 0.08
Zinc (Zn)	15.2 ± 0.3

Values are mean ± SD.

3.2 Physiological variables

The body weight of H rats was higher than C rats; body weight of HCL rats was lower than H rats. Lean mass was unchanged in all groups. Fat mass was highest in H rats followed by HCL, C and CCL rats. Food intake was higher in C rats compared to H rats. CCL rats had the same food intake as C rats whereas HCL rats ate less than H rats. C and CCL rats had lower energy intakes than H and HCL rats. CCL and HCL

rats had lower water intakes than C and H rats (Table 2). C rats had lower RER (Figure 1A) and heat production (Figure 1B) compared to H rats, while HCL rats were lower than H rats. C and CCL rats had lower RERs during the daytime.
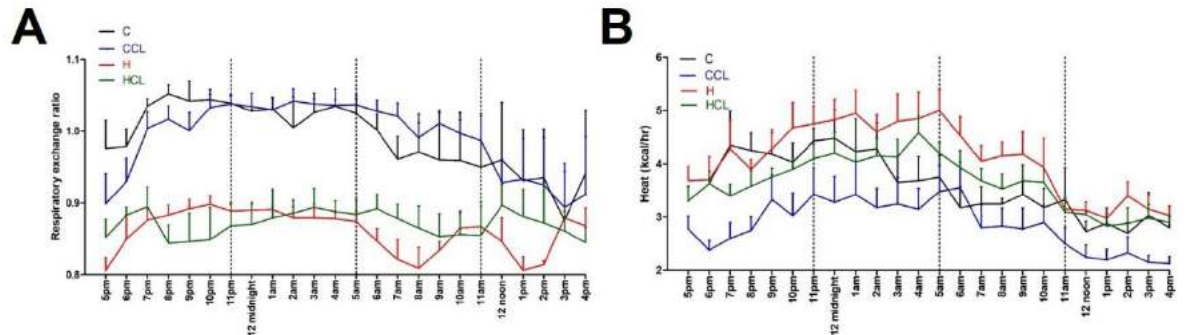

Figure 1. 24-hour indirect calorimeter Oxymax data. **Fig 1A** respiratory exchange ratio and **Fig 1B** heat production in corn starch diet-fed rats (C), corn starch diet-fed rats supplemented with 5% *C. lentillifera* (CCL), high-carbohydrate, high-fat diet-fed rats (H) and high-carbohydrate, high-fat diet-fed rats supplemented with 5% *C. lentillifera* (HCL).

Total abdominal fat was highest in H rats followed by HCL, C and CCL (Table 2). Plasma triglyceride concentrations were higher in H and HCL rats compared to C and CCL rats. Plasma total cholesterol concentrations were highest in H rats and similar in C, CCL and HCL rats (Table 2). Plasma non-esterified fatty acids were highest in H rats followed by HCL, C and CCL rats. C rats had lower basal blood glucose concentrations compared to H rats. None of the interventions reduced basal blood glucose concentrations (Table 2). The blood glucose area under the curve was not different between groups (Table 2).

After eight weeks, systolic blood pressure of H diet-fed groups (H and HCL) was higher than C diet-fed groups (C and CCL) (Table 2). Systolic blood pressure in H rats was higher at 16 weeks than in C rats. HCL rats had decreased systolic blood pressure compared to H control rats (Table 1). Diastolic stiffness was higher in H rats compared to C rats. HCL rats showed normalised left ventricular diastolic stiffness (Table 1). Left ventricular weights with septum and right ventricular wet weights were similar among all groups (Table 2).

Left ventricles from H rats showed infiltration of inflammatory cells and increased collagen deposition whereas these changes were not seen in left ventricles from C rats (Figure 2). Left ventricles from HCL rats showed decreased infiltration of

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inflammatory cells (Figure 2) and decreased collagen deposition compared to H rats (Figure 2). Livers from H rats showed increased fat deposition and infiltration of inflammatory cells compared to livers from C rats (Figure 2). HCL rats had reduced fat deposition compared to H (Figure 2). Plasma activities of ALT and AST were not different between the groups (Table 2).

3.3 Gut structure and microbiome

Histology of ileum and colon did not show any structural abnormalities in the experimental groups demonstrated by normal crypt depth, villi length and goblet cells and lack of inflammatory cell infiltration (Figure 2).

The gut microbiome was defined as the collective genomes of the microbes in the rat's colon. After quality filtering, there were a total of 788,078 sequences and these were clustered into 1282 zOTUs. The calculated rarefaction curves based on rarefied and unrarefied data as well as Good's coverage of $99.69 \pm 0.08\%$ revealed that the bacterial community was almost fully recovered by the surveying effort. There was no difference in Shannon's diversity between the four groups. There was higher richness in the HCL group and this difference was more pronounced for the HCL group compared to the C and CCL diets. There were differences between C and H groups indicating an effect of basal feed on the bacterial community structure (Figure 3). The addition of *C. lentillifera* (CCL and HCL) changed the bacterial communities.

Table 2. Responses to *Caulerpa lentillifera*

Variable	C	CCL	H	HCL	P value		
					Diet	**Treatment**	**D x T**
Physiological variables							
0 wk body weight	337 ± 1	339 ± 1	338 ± 1	337 ± 1	0.6196	0.6196	0.1408
8 wk body weight	364 ± 12^b	358 ± 6^b	439 ± 9^a	440 ± 8^a	<0.0001	0.7828	0.6997
16 wk body weight	411 ± 14^c	389 ± 6^c	573 ± 10^a	468 ± 10^b	<0.0001	<0.0001	0.0002
8 wk lean/fat mass proportion	5.9 ± 0.7^b	10.7 ± 2.6^a	2.6 ± 0.2^c	3.2 ± 1.2^c	0.0007	0.0744	0.1622
16 wk lean/fat mass proportion	4.2 ± 0.5^b	6.0 ± 0.7^a	1.3 ± 0.2^d	2.3 ± 0.4^c	<0.0001	0.0060	0.4137
16 wk lean mass (g)	316 ± 7	314 ± 5	303 ± 14	303 ± 9	0.2069	0.9155	0.9155
16 wk fat mass (g)	80 ± 9^c	57 ± 4^d	258 ± 28^a	148 ± 14^b	<0.0001	0.0002	0.0111
Visceral adiposity (%)	5.1 ± 0.2^c	4.3 ± 0.2^d	9.8 ± 0.8^a	7.7 ± 0.7^b	<0.0001	0.0115	0.2436
16 wk abdominal circumference (cm)	19.0 ± 0.4^c	18.4 ± 0.2^c	23.4 ± 0.4^a	21.1 ± 0.3^b	<0.0001	<0.0001	0.0149
Body mass index (g/cm^2)	0.67 ± 0.02^b	0.63 ± 0.01^b	0.77 ± 0.02^a	0.69 ± 0.01^b	<0.0001	0.0004	0.2126
Food intake 0-8 wk (g/day)	37.5 ± 1.4^a	37.6 ± 0.4^a	27.8 ± 1.7^b	25.9 ± 0.7^b	<0.0001	0.4469	0.3984
Food intake 9-16 wk (g/day)	39.7 ± 2.2^a	37.5 ± 0.9^a	29.3 ± 2.5^b	19.3 ± 1.0^c	<0.0001	0.0015	0.0353
Water intake 0-8 wk(g/day)	33.4 ± 2.8	30.2 ± 1.7	31.4 ± 3.1	29.1 ± 0.7	0.5005	0.2346	0.8446
Water intake 9-16 wk(g/day)	25.9 ± 3.3^b	52.6 ± 2.0^a	29.8 ± 2.1^b	50.8 ± 1.2^a	0.6470	<0.0001	0.2173
Energy intake 0-8 wk (kJ/day)	421 ± 16^b	422 ± 4^b	603 ± 34^a	598 ± 25^a	<0.0001	0.9301	0.8953
Energy intake 9-16 wk (kJ/day)	447 ± 24^b	416 ± 10^b	620 ± 46^a	551 ± 21^a	<0.0001	0.0856	0.5074
Cardiovascular variables							
8 wk systolic blood pressure (mmHg)	118 ± 6	120 ± 2	131 ± 2	126 ± 3	0.0083	0.6597	0.3075
16 wk systolic blood pressure (mmHg)	119 ± 3^b	117 ± 4^b	135 ± 3^a	118 ± 3^b	0.0356	0.0198	0.0617
Diastolic stiffness (κ, dimensionless)	21.1 ± 1.5^c	22.1 ± 1.2^c	30.2 ± 0.6^a	27.1 ± 0.8^b	<0.0001	0.3375	0.0649
Left ventricle collagen area (%)	7.8 ± 0.7^c	9.1 ± 1.2^c	29.4 ± 2.2^a	22.1 ± 2.8^b	<0.0001	0.2748	0.1482

Organ weights							
Liver (mg/mm)	235 ± 11^b	250 ± 6^b	403 ± 18^a	379 ± 13^a	<0.0001	0.7258	0.1332
Retroperitoneal fat (mg/mm)	231 ± 15^c	178 ± 13^d	628 ± 68^a	398 ± 37^b	<0.0001	0.0010	0.0320
Epididymal fat (mg/mm)	69 ± 6^c	41 ± 4^d	182 ± 32^a	134 ± 16^b	<0.0001	0.0432	0.5865
Omental fat (mg/mm)	149 ± 14^c	134 ± 7^c	325 ± 39^a	229 ± 31^b	<0.0001	0.0392	0.1280
Total abdominal fat (mg/mm)	450 ± 27^c	353 ± 21^d	1136 ± 135^a	761 ± 77^b	<0.0001	0.0049	0.0876
Metabolic variables							
Total cholesterol (mmol/L)	1.59 ± 0.06^b	1.54 ± 0.05^b	1.73 ± 0.09^a	1.56 ± 0.06^b	0.2368	0.1063	0.3733
HDL-cholesterol (mmol/L)	0.93 ± 0.14^a	1.11 ± 0.07^a	0.95 ± 0.09^a	0.70 ± 0.05^b	0.0268	<0.0001	<0.0001
LDL-cholesterol (mmol/L)	0.55 ± 0.14	0.80 ± 0.32	0.92 ± 0.08	0.61 ± 0.07	0.7120	0.9020	0.2551
Triglycerides (mmol/L)	0.50 ± 0.05^b	0.64 ± 0.10^b	1.15 ± 0.13^a	1.12 ± 0.18^a	<0.0001	0.6603	0.4977
Non-esterified fatty acids (mmol/L)	0.68 ± 0.12^c	0.47 ± 0.04^d	2.71 ± 0.29^a	1.61 ± 0.49^b	<0.0001	0.0298	0.1342
ALT (U/L)	39 ± 5	39 ± 6	50 ± 11	62 ± 12	0.0663	0.5098	0.5098
AST (U/L)	138 ± 20	163 ± 14	174 ± 17	220 ± 45	0.0917	0.1949	0.6989
Liver inflammatory cells (cells/200µm^2)	12 ± 2	13 ± 2	28 ± 2	21 ± 3	0.0001	0.2859	0.1576
Liver fat vacuole area (µm^2)	11.2 ± 1.9^c	9.2 ± 1.4^c	84.5 ± 2.6^a	45.5 ± 3.1^b	<0.0001	<0.0001	<0.0001
Oral glucose tolerance test							
0 wk basal glucose (mmol/L)	2.8 ± 0.1	2.8 ± 0.1	2.9 ± 0.1	2.8 ± 0.1	0.6196	0.6196	0.6196
0 wk AUC (mmol/L/120 minutes)	648 ± 31	641 ± 19	620 ± 44	633 ± 19	0.5526	0.9210	0.7411
8 wk basal glucose (mmol/L)	2.5 ± 0.2	2.5 ± 0.1	3.2 ± 0.1	3.4 ± 0.2	<0.0001	0.5304	0.5304
8 wk 120 min glucose (mmol/L)	4.0 ± 0.1	3.7 ± 0.1	4.8 ± 0.4	5.3 ± 0.1	<0.0001	0.6486	0.0732
8 wk AUC (mmol/L/120 min)	526 ± 24	566 ± 15	613 ± 23	688 ± 14	<0.0001	0.0052	0.3751
16 wk basal glucose (mmol/L)	2.9 ± 0.2	2.4 ± 0.1	2.8 ± 0.2	2.8 ± 0.1	0.3480	0.1210	0.1210
16 wk 120 min glucose (mmol/L)	3.4 ± 0.1	3.3 ± 0.1	4.7 ± 0.3	4.5 ± 0.1	<0.0001	0.3912	0.7742
16 wk AUC (mmol/L/120 min)	478 ± 17	472 ± 11	563 ± 16	571 ± 9	<0.0001	0.9505	0.6643

Insulin tolerance test							
8 wk ITT 120 min glucose (mmol/L)	3.3 ± 0.9	2.7 ± 0.5	3.0 ± 0.4	4.3 ± 0.2	0.2531	0.5361	0.0976
8 wk ITT AUC (mmol/L/120 min)	147 ± 45	144 ± 22	353 ± 40	441 ± 20	<0.0001	0.2122	0.1823
16 wk ITT 120 min glucose (mmol/L)	1.1 ± 0.4	2.3 ± 0.5	3.6 ± 0.3	3.4 ± 0.3	<0.0001	0.1997	0.0752
16 wk ITT AUC (mmol/L/120 min)	111 ± 11	207 ± 28	356 ± 36	332 ± 28	<0.0001	0.1944	0.0334

Values are presented as mean ± SEM, n = 10-12. Means in a row with superscripts without a common letter differ (a, b, c or d), $p < 0.05$. C, corn starch diet-fed rats; CCL, corn starch diet-fed rats supplemented with 5% *C. lentillifera*; H, high-carbohydrate, high-fat diet-fed rats; HCL, high-carbohydrate, high-fat diet-fed rats supplemented with 5% *C. lentillifera*. ALT, alanine transaminase; AST, aspartate transaminase; AUC, area under the curve; ITT, insulin tolerance test.
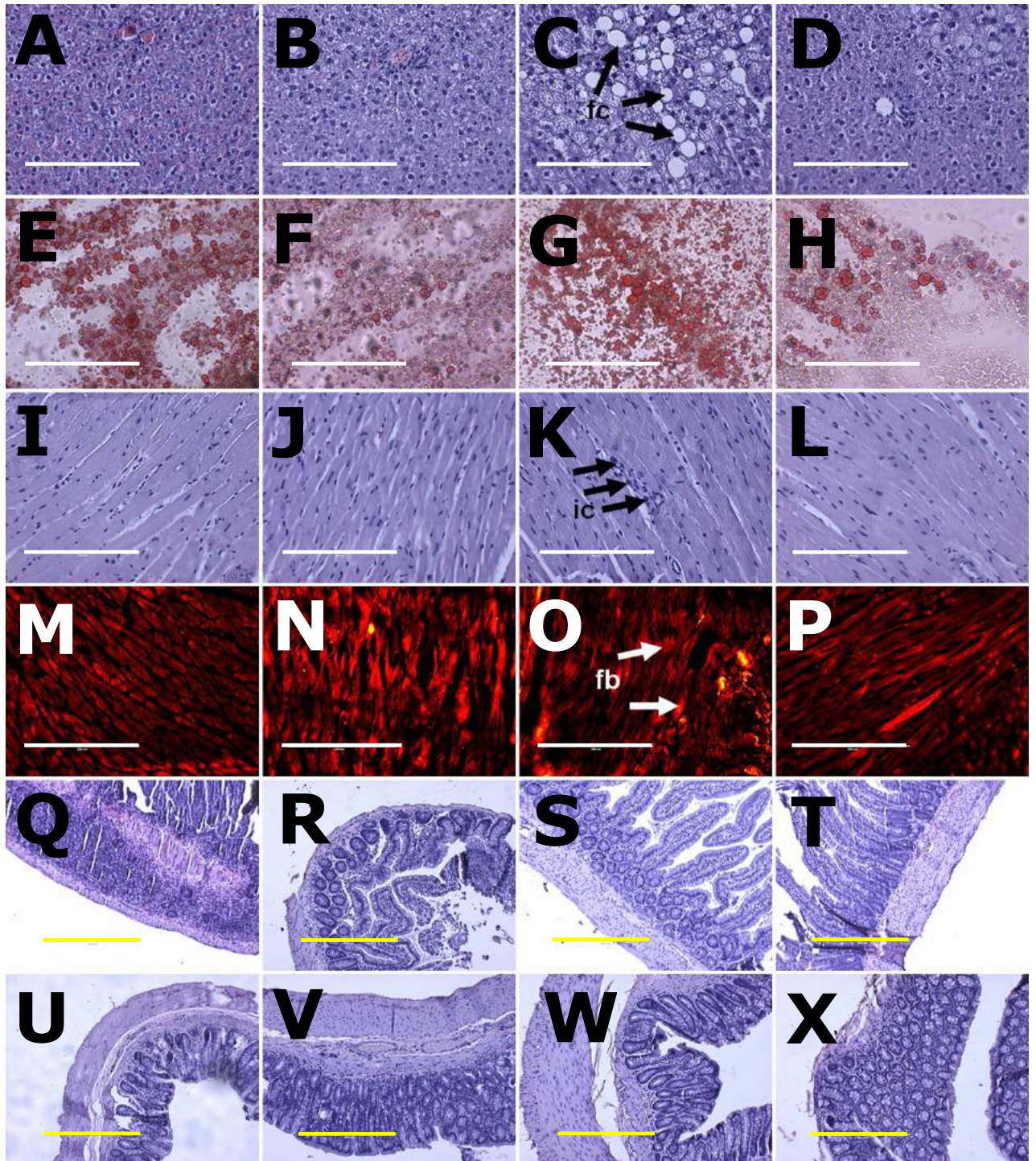
Figure 2. Liver fat using haematoxylin and eosin stain (A-D) and oil red O stain (E-H); heart inflammation (I-L) using haematoxylin and eosin stain; heart fibrosis (M-P) using picrosirius red stain; ileum (Q-T) and colon (U-X) structure using haematoxylin and eosin stain. Each row is in the following order: corn starch diet-fed rats (C), corn starch diet-fed rats supplemented with 5% *C. lentillifera* (CCL), high-carbohydrate, high-fat diet-fed rats (H) and high-carbohydrate, high-fat diet-fed rats supplemented with 5% *C. lentillifera* (HCL). Fat cells=fc; inflammatory cells=ic; fibrosis=fb. Scale bar is 200 μm for A-P (20x) and 100 μm for Q-X (10x).
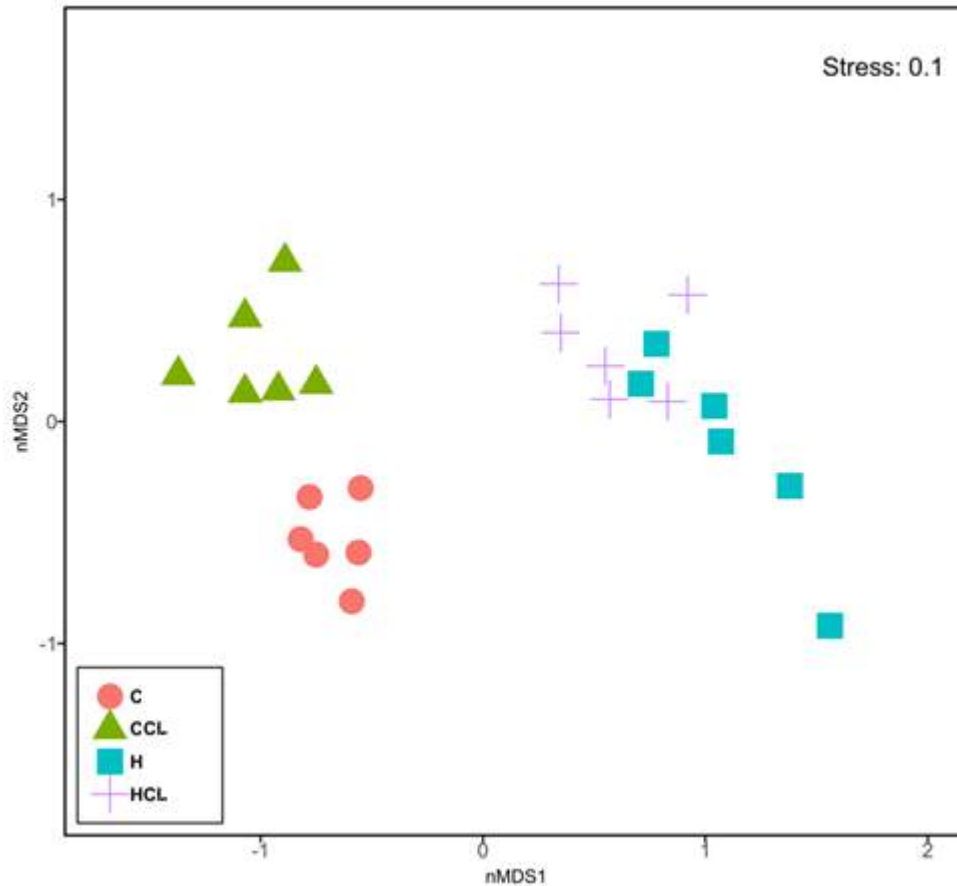
Figure 3. MDS plot of bacterial communities structure of faecal samples from different diets. Corn starch diet-fed rats (C), corn starch diet-fed rats supplemented with 5% *C. lentillifera* (CCL), high-carbohydrate, high-fat diet-fed rats (H) and high-carbohydrate, high-fat diet-fed rats supplemented with 5% *C. lentillifera* (HCL).

4. Discussion

C. lentillifera was chosen over other *Caulerpa* species such as *C. racemosa* because of its better growth in Australian tropical waters, potential commercial role and lower accumulation of toxic metals (5, 12). The product was bought from a supplier in Vietnam because this is currently a commercial product and thus represents the seaweed market. *C. lentillifera* supplementation improved cardiometabolic risk factors in HCL rats by decreasing body weight by ~20% compared to H rats, reducing systolic blood pressure, reducing diastolic stiffness constant, and reducing plasma total cholesterol and non-esterified fatty acid concentrations. The gut microbiome of HCL rats was different to H rats, and closer to CCL rats.

Caulerpa species are traditionally used in the Indo-Pacific area as fresh vegetables due to their palatable taste, their availability, their nutritional properties and

people's general awareness of natural products (29). The total annual crop harvested in Fiji, Samoa and Tonga Islands in 2012 was 123 tonnes of wet material worth 266,492 USD (30). Compounds of *C. lentillifera* include sulphated polysaccharides, sterols and proteins (31) as well as the secondary metabolites flavonoids, caulerpin and caulerpenyne (32). Caulerpin is a bis-indole alkaloid isolated from the marine green seaweed *Caulerpa* and red seaweed *Chondria armata* (33). It is potentially bioactive with a wide range of therapeutic activities including anti-diabetic (34), anti-inflammatory and antinociceptive properties (35). The mineral content (in % of dry weight) of three *Caulerpa* species tested varied marginally with Na the highest followed by K, Ca and Mg (36). *C. lentillifera* from the current study contained (% of dry weight) 44% carbohydrates which is similar to literature values of 37-49%, 14% lipids (literature 2-3%) which is 5-6 times higher and 7% protein (literature 7-13%) which is similar (36). This study investigated the whole seaweed and it was likely that physiological and metabolic responses in the rats was due to the combination of bioactives and thus had an additive effect.

C. lentillifera polysaccharides are sulphated and hence we investigated whether well-known sulphated polysaccharides from this green seaweed have similar effects to those from red seaweed against metabolic syndrome as our research group (37) and others have reported (38). Sulphated polysaccharides from *Caulerpa* species are found at high concentrations in marine seaweeds and their complex and heterogeneous structure of repetitive sugars units have been studied (39). It is likely that these compounds are involced in mechanical, ionic and osmotic regulation, favouring the survival of these organisms in the marine environment (39). A neutral glucan and an acidic heteroglycan sulphate containing arabinose, xylose and galactose residues were isolated from *C. racemosa*. The major polymer was xyloarabinogalactan, which is sulphated (40).

Caulerpa species have shown anti-obesity, anti-diabetic, anti-hypertensive, anti-inflammatory, anti-nociceptive and anti-tumour responses (33). The reduced inflammation in the heart and liver may be due to an immunostimulatory effect. *C. lentillifera* polysaccharides have enhanced immunostimulatory activity in immunosuppressed mice and modulated gut microbiota (11). Chronic inflammation in adipose tissue likely plays a crucial role in the development of obesity-associated insulin resistance (41). Recently, the role of innate lymphoid cells in mediating obesity-associated inflammation has been of interest (42, 43). Consumption of a high-

fat diet leads to increased concentrations of gut inflammatory cytokines such as TNF-α, IL-1β and IL-12 which are related to weight gain, adiposity, and increased plasma insulin and glucose concentrations (44). An intervention that reduces gut and systemic inflammation may lead to decreased obesity-associated conditions such as metabolic syndrome.

Caulerpa polysaccharides undergo minimal digestion in the stomach and fermentation by colonic bacteria (45), hence meeting the definition of prebiotics (46). Health benefits of prebiotics include decreased blood pressure and body weight (14, 47) similar to the responses from the current study. Using the same rat model of diet-induced obesity, a prebiotic mixture of inulin and oligofructose was an effective dietary fibre reducing body weight gain, plasma concentrations of free fatty acids and triglycerides, and systolic blood pressure, and attenuating inflammatory cell infiltration in the heart and liver (48).

Increasingly, there are studies reporting the health benefits from seaweed extracts (49) which may be attributed to their unique nutritional profile of polyunsaturated fatty acids, pigments and trace minerals (12). Hypotheses surrounding mechanisms for green algae in improving health revolve around prebiotic effects from their high-fibre content. *C. lentillifera* from the current study contained 17.5% total dietary fibre of which 16.6% was insoluble fibre which may have resulted in an increased colonic production of short chain fatty acids (SCFA) including acetic, propionic and butyric acids as the most common (50). Almost all of the fibre was insoluble which is not converted to energy and improves faecal bulking and thus increases satiety (51).

Beneficial physiological effects, including cardiovascular improvements such as blood pressure regulation, are controlled by the gut microbiome and mediated by SCFA (52) while SCFA promote a lean and healthy phenotype (53, 54). Polysaccharides exert their action through a wide range of mechanisms including selective fermentation, lowering the gut pH, faecal bulking, preventing gut colonisation by pathogens, controlling putrefactive bacteria, and therefore reducing the host's exposure to toxic metabolites (55). These effects are likely due to dietary fibre increasing SCFA production as SCFA are used as an energy source by selected gut microbiota. SCFA decrease luminal pH, improve calcium and magnesium absorption, reduce potential pathogenic bacteria and act as an energy source for epithelial cells (56, 57).

There is scarce information on global seaweed consumption, with data from Japan the most reliable. Daily seaweed consumption per person in Japan has remained relatively consistent over the last 40 years (4.3 g/day in 1955 and 5.3 g/day in 1995) (58). In the current study, HCL rats consumed ~0.97 ± 0.01 g/day 5% *C. lentillifera* for the final eight weeks of the protocol. Using the Reagan-Shaw calculation for rat-to-human scaling (59), there is a 6-fold increase in dosage related to body weight from humans to rats. Hence humans would need to consume 5.8 g/day for an equivalent dose from the current study, which is realistic based on seaweed intakes from Japan.

Aquaculture is a sustainable way of producing tropical seaweeds for human food production and for biodiversity enrichment with the potential to enhance fisheries (60). Fresh seaweed can be used in salads, fruit and vegetable juices, re-hydrated seaweed can be used in a variety of dishes with rice and beans and dried seaweed can be used as substitute for wheat and maize flours for baking and cooking (60). There are a variety of recipes available for *C. lentillifera*, with popular dishes including green caviar, named due to its similar appearance to fish eggs (61). *C. lentillifera* is widely consumed throughout the Pacific Islands and south-east Asia (30). *C. lentillifera* has been cultivated in 1m^2 trays to control quality and increase the accessibility of the crop during harvesting periods (30). Another *Caulerpa* species, *C. racemosa*, accumulated minerals from the water in which it grows, including toxic metals such as lead, mercury and arsenic in dangerously high concentrations (62). Hence, we have used *C. lentillifera* which does not accumulate these metals.

The moisture content of fresh seaweed is very high, up to 94% of the biomass (49). Consequently, in this study we used dried biomass to determine the effects of the non-water compounds, such as fibre for their prebiotic effects and pigments such as chlorophyll and *β*–carotene for anti-oxidant or anti-inflammatory effects. In mice, 5 mg/kg body mass/day of chlorophyllin derived from chlorophyll in the drinking water for eight weeks attenuated intestinal and hepatic inflammation and ameliorated liver fibrosis (63) possibly by inhibiting the NF-κB pathway and modulating gut microbiota. Approximately 59% of gut bacteria were *Bacteroidetes*, while 14% were *Firmicutes* in both control and chlorophyllin-supplemented mice. In contrast, in the fibrotic mice, the population was changed, showing decreased *Bacteroidetes* as 29% and increased *Firmicutes* as 40% of total bacterial content (63). An inverse association between carotenoid intake and metabolic syndrome was reported in a systematic review and meta-analysis (64). Fucoxanthin from seaweed has anti-obesity, anti-

oxidant and anti-inflammatory effects (65). By using the whole seaweed, in the form it is currently readily available as, we suggest that all components are working together for the observed responses in the rats.

Other uses for cultivation of *Caulerpa* species include using an aqueous extract from *C. racemosa* to synthesise nanoparticles in an eco-friendly and cost-effecive manner (66). Additionally, an extract from *C. serrulata* has been used as a sustainable alternative to more complicated procedures for chemical nanoparticle production (67).

Caulerpa species contain bioactives which have biological activity against metabolic syndrome parameters. Large-scale cultivation can provide human and animal food, improve biodiversity and provide extracts for biotechnological applications. Although currently only widely consumed in south-east Asia and the Pacific, there is great incentive for Western countries to cultivate and consume *C. lentillifera*.

5. Conclusions

C. lentillifera supplementation improved the health of HCL rats by decreasing body weight by ~20%, reducing systolic blood pressure, reducing diastolic stiffness constant, reducing plasma total cholesterol and non-esterified fatty acid concentrations compared to H rats. Consuming *C. lentillifera* in a functional food capacity (low dose) did not appear to have any negative consequences. The additive effect from the combination of bioactive compounds was the likely mechanism for the responses in the rats. Further investigation into potential mechanisms of action may provide additional evidence for *C. lentillifera* as a functional food.

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CHAPTER 6. MICROALGAE IN DIET-INDUCED METABOLIC SYNDROME

The previous chapters have investigated health benefits from red, brown and green macroalgae. This chapter changes the focus to microalgae and aims to determine if *Nannochloropsis oceanica* can attenuate symptoms of diet-induced metabolic syndrome in rats. Microalgae are considered part of a healthy diet as they contain fatty acids, proteins, amino acids, pigments, vitamins and minerals. Traditionally, microalgae are fed to fish such as salmon, mullet and mackerel to provide polyunsaturated fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). However, for humans, marine fatty fish are the main sources of EPA and DHA. Studies that have established the cardiovascular and metabolic benefits of EPA are based primarily on the Mediterranean diet, with a relatively high intake of olive oil and fatty fish, but microalgae can also provide a sustainable source of EPA, up to 7% of dry weight. Further, in recent years, there has been an increased interest in microalgae for biotechnological applications such as aquaculture, fish food, livestock feeds, health foods, biofuels and wastewater treatment so commercially viable production is now feasible. Microalgae grow well in South-East Queensland, Australia and this area may be a key location to provide good quality microalgae in sustainable amounts for Australian and international use. Bio-refineries have been developed to exploit the rapid growth and high oil productivity of microalgae by novel harvesting and extraction techniques. My hypothesis in this study is that *N. oceanica* grown in South-East Queensland could reduce the signs of diet-induced metabolic syndrome in rats because of the presence of EPA in the microalgae.

The microalga Nannochloropsis oceanica can be used as a food intervention in diet-induced metabolic syndrome in Wistar rats

Ryan du Preez [1,2], Lindsay Brown [1,2], Marwan Majzoub [3], Torsten Thomas [3], Sunil K Panchal [1,*]

[1]Functional Foods Research Group, University of Southern Queensland, Toowoomba, QLD 4350, Australia

[2]School of Health and Wellbeing, University of Southern Queensland, Toowoomba, QLD 4350, Australia

[3]Centre for Marine Bio-Innovation, University of New South Wales, Sydney, NSW 2052, Australia

*Correspondence: Sunil.Panchal@usq.edu.au; Tel.: +61 7 4631 1012

Abstract

The microalga, *Nannochloropsis oceanica*, contains the omega-3 polyunsaturated fatty acid, eicosapentaenoic acid (EPA), at up to 9% of dry weight. This study investigates a potential role of this sustainable EPA source in the reversal of metabolic syndrome. Male Wistar rats (n = 48) were divided into four groups in a 16-week protocol. Two groups were fed either corn starch or high-carbohydrate, high-fat diets (C and H, respectively) for the full 16 weeks. The other two groups received C and H diets for eight weeks and then received *N. oceanica* in these diets for the final eight weeks (CN and HN, respectively) of the protocol. The H diet was high in fructose and sucrose, together with high saturated and *trans* fats. H rats developed obesity, hypertension, dyslipidaemia, fatty liver disease and increased left ventricular fibrosis. *N. oceanica* increased lean mass in CN and HN rats, possibly due to the increased protein intake, and decreased fat mass in HN rats. Cardiovascular, liver and metabolic parameters, and gut bacterial community structure were unchanged. A limiting factor was the intactness of the cell wall of *N. oceanica*, which may have decreased the bioavailability of both EPA and chlorophyll. We conclude that cell membrane disruption of *N. oceanica* is essential for optimal biological activity.

Keywords: *Nannochloropsis oceanica*, microalgae, metabolic syndrome, gut microbiome, eicosapentaenoic acid, chlorophyll

1. Introduction

Microalgae are unicellular organisms that, in the presence of sunlight, convert carbon dioxide into biomass containing high-value products such as fatty acids and carotenoids (1). *Nannochloropsis* species are microalgae that belong to the class of Eustigmatophyceae which is recognised for high photoautotrophic biomass productivity, a natural ability to accumulate lipids and successful cultivation at industrial scale (2). The *Nannochloropsis* genus comprises six species (*gaditana, salina, oculata, granulata, oceanica* and *limnetica*) (3). *Nannochloropsis* species on average contain (in % of dry weight) 37.6% carbohydrates, 28.8% crude protein and 18.4% total lipids (4) with violaxanthin and chlorophyll a as the main pigments (5). Selected microalgae are sustainable sources of long-chain polyunsaturated fatty acids (PUFA) with *Nannochloropsis* species containing only eicosapentaenoic acid (EPA, 20:5 *n*−3; (6)) while other microalgae such as *Isochrysis* and *Schizochytrium* species produce predominantly docosahexaenoic acid (DHA, 22:6 *n*−3; (7)). Consumption of PUFA may lead to decreased incidence of obesity and its associated diseases through their anti-inflammatory and anti-thrombotic properties as well as improving the blood lipid profile (8-10). For example, a meta-analysis found that a higher circulating *n*−3 PUFA concentration was associated with a lower metabolic syndrome risk (8).

Marine fatty fish such as salmon, mullet and mackerel are the main sources of EPA and DHA for human consumption (11, 12). However, due to the excessive and sometimes poorly regulated fishing industry, the depletion of worldwide fish stocks is straining the sustainability of production of *n*−3 long-chain PUFA (13). In contrast, microalgae can be used for sustainable production of *n*−3 PUFA (14) and so can be an important source for farmed fish (15). Microalgae grow well in south-east Queensland, Australia (16) and hence this may be a key location to provide good quality microalgae for Australian and international use. The biomass from *Nannochloropsis* species also contains high-value products such as other fatty acids, sterols and carotenoids with applications in food, cosmetic and pharmaceutical industries (1). The content of EPA in *N. oceanica* can be regulated by environmental changes; as an example, EPA content was doubled to ~7% through exposure to ultraviolet C (100-280 nm) at a dose of 100 mJ/cm^2 for 24 hours (17).

In this study, *N. oceanica* farmed locally under controlled conditions was freeze-dried and fed to rats with diet-induced metabolic syndrome to determine cardiovascular, liver and metabolic health benefits. A validated diet-induced rat model

of metabolic syndrome that closely mimics the symptoms of human metabolic syndrome was used. We measured systolic blood pressure, diastolic stiffness, cardiac inflammatory cells and collagen deposition in the heart for cardiovascular effects; plasma liver enzyme activities, inflammatory cells and fat vacuoles in the liver for liver effects; body weight, total cholesterol and triglyceride concentrations and glucose and insulin tolerance tests for metabolic effects. Further, as functional foods may reverse obesity-induced changes in the gut microbiome (18, 19), we characterised the changes in its composition after seaweed treatment. We hypothesised that 5% *N. oceanica* supplementation for the last eight weeks of the protocol will reverse the changes induced by the high-carbohydrate, high-fat diet. The mechanisms of these hypothesised effects could include the actions of EPA, chlorophyll a and protein to prevent infiltration of inflammatory cells into organs such as the heart and liver and provide an increased lean mass. However, as reported in the literature, a key feature of microalgae are the rigidity of their cell wall which could limit the bioavailability of nutrients; hence, other studies have used several cell disruption methods such as mechanical, physical, chemical and enzymatic approaches (20) or solvent extraction (21). For example, a laboratory ball-mill effectively disrupted the cell wall which enabled the protein and fatty acids to become bioavailable to mice (22). In our study, the cell wall was not disrupted or extracted allowing the measurement of responses to intact microalgae.

2. Materials and Methods

2.1 *N. oceanica* source

40 L of *N. oceanica* paste was harvested from the Teraform microalgae farm (http://teraform.com.au/), Miles, QLD, Australia during June and July 2018. The paste was transported frozen to the University of Southern Queensland, Toowoomba, QLD, Australia, in sealed plastic buckets. The paste was kept frozen until it was freeze-dried (Martin Christ Alpha 2–4 LD plus, John Morris Scientific, QLD, Australia). 5 L batches were freeze-dried for 36-48 hours at 0.011 mbar and -60°C. The powder was stored at 4°C in sealed plastic containers until processing or experiments.

2.2 Rats and diets

All experimental protocols were approved by the Animal Ethics Committee of the University of Southern Queensland (approval number 17REA010) under the guidelines of the National Health and Medical Research Council of Australia. Male Wistar rats (8-9 weeks old; 337 ± 1 g, n = 48) were obtained from the Animal Resource Centre, Murdoch, WA, Australia. Rats were individually housed in a temperature-controlled (21±2ºC), 12-hour light/dark conditions with unrestricted access to food and water. Rats were randomly allocated to four groups, each with 12 rats. Two groups were fed either corn starch or high-carbohydrate, high-fat diets (C and H, respectively) (23) for the full 16 weeks. The other two groups received C and H diets for eight weeks and then received 5% dried *N. oceanica* mixed in the food for the final eight weeks (CN and HN, respectively).

2.3 Measurements before euthanasia

A mediquip anaesthesia machine was used to induce light sedation to immobilise the rat. Rats were pre-oxygenated with 2 L/min for three minutes prior to 5% isoflurane induction vaporised in 1.5 L/min. Rats were placed in a nose mask and maintained with 1.5% isoflurane in 1.5 L/min O_2 for the duration of each procedure. Systolic blood pressure measurements were performed after 8 and 16 weeks of feeding using an MLT1010 Piezo-Electric Pulse Transducer (ADInstruments, Bella Vista, NSW, Australia) and an inflatable tail-cuff connected to an MLT844 Physiological Pressure Transducer (ADInstruments) connected to a PowerLab data acquisition unit (ADInstruments). Dual-energy X-ray absorptiometry was performed on all rats after 8 and 16 weeks of feeding using a Norland XR46 DXA instrument (Norland Corp., Fort Atkinson, WI, USA). Scans were analysed using the manufacturer's recommended software for use in laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1; Norland Corp. (23).

Oral glucose tolerance tests were performed on rats after overnight deprivation of food. Fructose-supplemented drinking water in H and HN rats was replaced with normal drinking water. Basal blood glucose concentrations were determined in tail vein blood using Medisense Precision Q.I.D. glucometer (Abbott Laboratories, Bedford, MA, USA) and glucose test strips (Freestyle Optium Blood Glucose Test Strips, Abbott Diabetes Care Ltd, Sydney, NSW, Australia). Rats were then given 2 g/kg body weight of glucose as a 40% (w/v) aqueous solution by oral gavage.

Following this, blood glucose concentrations were measured at 30, 60, 90 and 120 minutes following glucose administration (23).

Insulin tolerance tests were performed on rats after deprivation from food for four hours. Fructose-supplemented drinking water in H and HN rats was replaced with normal drinking water. Basal blood glucose concentrations were determined in tail vein blood using Medisense Precision Q.I.D. glucometer (Abbott Laboratories, Bedford, MA, USA) and glucose test strips (Freestyle Optium Blood Glucose Test Strips, Abbott Diabetes Care Ltd, Sydney, NSW, Australia). Rats were then given 0.66 IU/kg of insulin (Eli Lilly Australia, West Ryde, Australia) by intraperitoneal injections. Following this, blood glucose concentrations were measured at 30, 60, 90 and 120 minutes following insulin administration (24). If blood glucose concentrations dropped below 1.1 mmol, 4 g/kg body weight of glucose as a 40% (w/v) aqueous solution was immediately administered by oral gavage to prevent hypoglycaemia.

2.4 Measurements after euthanasia

Terminal anaesthesia was induced in rats by intraperitoneal injection of Lethabarb (pentobarbitone sodium, 100 mg/kg; Virbac, Peakhurst, NSW, Australia). After rats were anaesthetised, heparin was administered (200 IU) into the right femoral vein. The abdomen was then opened and blood (~6 mL) was withdrawn from the abdominal aorta, collected into heparinised tubes and centrifuged at $5000 \times g$ for 10 minutes. Plasma from each rat was stored at -20°C before analysis. Plasma samples collected during terminal experiments were used to test for enzyme activities and plasma concentrations of biochemical markers. Plasma activities of alanine transaminase (ALT) and aspartate transaminase (AST), and plasma concentrations of total cholesterol, triglycerides and non-esterified fatty acids were determined using appropriate kits and controls (23).

Hearts were removed for use in the isolated Langendorff heart preparation. Hearts were perfused with a modified Krebs-Henseleit bicarbonate buffer bubbled with 95% O_2–5% CO_2 and maintained at 35°C (23). Isovolumetric ventricular function was measured by inserting a latex balloon catheter into the left ventricle connected to a Capto SP844 MLT844 physiological pressure transducer and Chart software on a MacLab system. Left ventricular end-diastolic pressure values were measured during pacing of the heart at 250 beats/minute using an electrical stimulator. End-diastolic pressures were obtained from 0 to 30 mmHg for calculation of diastolic stiffness

constant (κ, dimensionless) (23). After completing the Langendorff heart measurements, hearts were separated into right ventricle and left ventricle with septum for weighing. Livers and abdominal fat pads (retroperitoneal, epididymal and omental) were isolated and weighed. These organ weights were normalised relative to the tibial length at the time of their removal (in mg of tissue/mm of tibial length) (23).

Two rats from each group were exclusively used for histological analysis. Tissues were also collected from two other rats in each group. Approximately 5-7 minutes after euthanasia, heart, liver and intestinal tissues were collected and fixed in 10% neutral buffered formalin for three days. The samples were then dehydrated and embedded in paraffin wax. Two slides were prepared for each heart and liver specimen and two random, non-overlapping fields per slide were taken to avoid biased analysis. Approximately 5 μm sections of the samples were cut using a microtome and stained with haematoxylin and eosin for determination of inflammatory cell infiltration and presence of liver fat vacuoles. In addition, liver portions were fixed in Tissue-Tek O.C.T. Compound (ProSciTech, Thuringowa, QLD, Australia) and stored at -20°C. Approximately 10 μm sections of frozen liver in O.C.T. media were cut using a cryostat and stained with Oil Red O to evaluate fat vacuoles. Collagen distribution was defined in the heart with picrosirius red stain. EVOS FL Colour Imaging System (v1.4 (Rev 26059); Advanced Microscopy Group, Bothell, WA, USA) was used to capture images (23). NIH ImageJ software (https://imagej.nih.gov/ij/) was used to quantify collagen deposition in heart sections, calculate liver fat vacuole area and to count inflammatory cells in liver sections.

Immediately after euthanasia and organ removal, two to three faecal pellets were collected from the colon of rats and stored at -80°C in nuclease-free tubes. DNA extraction and diversity profiling were performed by the Centre for Marine Bio-Innovation, University of New South Wales (Kensington, NSW, Australia). For DNA extraction of microbial samples, total microbial community DNA was extracted from faecal samples using the DNeasy Powersoil Kit (Qiagen Australia, Chadstone, VIC, Australia) following the manufacturer's instructions and following the protocol (25). For 16S rRNA gene amplification and sequencing, bacterial communities from faecal samples were investigated by sequencing 16S rRNA gene amplicons. The primers, 341F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGN GGCWGCAG) and 785R (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATC

TAATCC) were used to amplify the V3-V4 regions of the 16S rRNA gene. The reaction mixture (50 μL total volume per sample) consisted of Econotaq® PLUS GREEN 2X Master Mix (Astral Scientific, Gymea, NSW, Australia) (25 μL), Ambion® nuclease-free water (17 μL), the primer pair 341F and 785R (1.5 μL of each; 10 μM) and DNA template (5 μL). The PCR program consisted of an initial denaturation at 94°C (2 min), followed by 35 cycles of denaturation at 94°C (30 s), annealing at 55°C (30 s), extension at 72°C (40 s) and a final extension at 72°C (7 min). PCR products were then quantified using gel electrophoresis. Paired-end sequencing (2 x 300 bp) of the resulting 16S rRNA gene amplicons was performed at the Ramaciotti Centre for Genomics, University of New South Wales on a Illumina MiSeq platform following the MiSeq System User Guide (26). For 16S rRNA gene sequencing analysis, sequence data were initially quality-filtered and trimmed using Trimmomatic version 0.36 truncating reads if the quality dropped below 20 in a sliding window of 4 bp (27). USEARCH version 11.0.667 (28) was used for further processing (29) to merge and quality-filter sequencing reads, excluding reads with < 250 or > 550 nucleotides, in addition to reads with more than one ambiguous base or an expected error of more than 1. Filtered sequences were denoised and clustered into unique sequences (zero-distance operational taxonomic units; zOTUs) using the UNOISE algorithm (30) implemented in USEARCH. zOTUs represent unique bacterial entities and roughly are equivalent to species or strains. Chimeric sequences were removed *de novo* during clustering and subsequently in reference mode using UCHIME (31) with the SILVA database (https://www.arb-silva.de/browser/) (SILVA SSURef 132 NR) as a reference (32). zOTUs were then taxonomically classified (i.e., assigned a likely taxonomic name) by BLASTN (33) against the SILVA database. All non-bacterial OTUs were removed along with non-BLAST aligned and singleton OTUs. Finally, processed sequences were mapped on OTU sequences to calculate the distribution and counts of each OTU in every sample. Only OTUs occurring in more than two samples were considered for further statistical analysis.

2.5 Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Metabolic and physiological results were tested for variance using Bartlett's test and variables that were not normally distributed were transformed using log10 function prior to statistical analyses. Data from the four groups were tested by two-way analysis of

variance. When the interactions and/or the main effects were significant, means were compared using the Newman-Keuls multiple comparison *post hoc* test. Where transformations did not result in normality or constant variance, a Kruskal-Wallis non-parametric test was performed. A *p* value of <0.05 was considered as statistically significant. All statistical analyses were performed using Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

For microbiome results, rarefaction curves were generated using the *rarecurve* function in *vegan* (34) and used to determine if a complete representation of the sample's microbiome had been achieved given the sequencing effort. Prior to further analysis, the numbers of sequences were standardised across samples to account for different sequencing depths by randomly subsampling each sample to the lowest number of sequences counts obtained for any given sample (i.e., 14,908 counts). Bacterial alpha-diversities (i.e., zOTU richness and Shannon's diversity) were calculated in R (version 3.5.3) using the *rrarefy* function in the *vegan* package for community ecology analysis (35). A one-way ANOVA test in GraphPad Prism 8.0.2 (San Diego, CA, USA) followed by Tukey's pairwise comparisons test was used to determine the significance between the different groups, a *p* value of <0.05 was considered as statistically significant.

For multivariate analysis of bacterial communities, OTU tables were imported into PRIMER (36) to compare the community structure (i.e., relative abundance data). Bray-Curtis similarity coefficients were calculated using square-root transformed OTU abundances and the resulting similarity matrix was visualised using non-metric, multi-dimensional scaling (nMDS). Permutational multivariate analysis of variance (PERMANOVA) (37) with 9999 random mutations was used to test the effect of treatment on bacterial communities in rat faecal samples. 'Treatment' was set with corn-starch diet (C), corn-starch diet supplemented with *N. oceanica* (CN), high-carbohydrate, high-fat diet (H) and high-carbohydrate, high-fat diet supplemented with *N. oceanica* (HN) as fixed factors.

3. Results

3.1 N. oceanica cell wall and nutritional composition

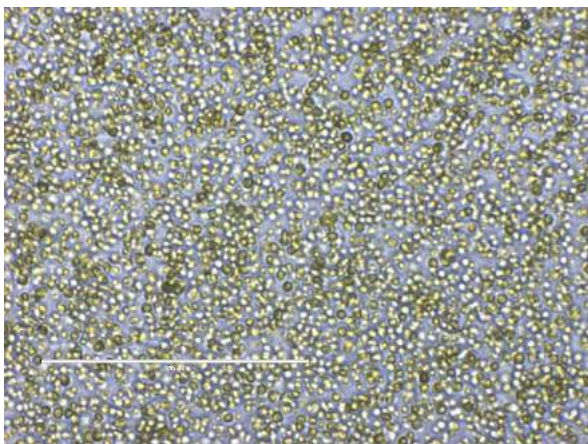The cell wall of *N. oceanica* was intact as shown in Figure 1.

Figure 1. *N. oceanica* showing an intact cell wall in brightfield microscopy. Scale bar is 100 μm.

3.2 Physiological variables

The body weight of H rats was higher than C rats. The body weight of HN rats was the same as H rats, while CN rats were higher than C rats. Lean mass was the same in C and H rats. In CN and HN rats, the lean mass was higher than their respective controls. Fat mass was higher in H rats compared to C rats. CN rats had the same fat mass than C rats, HN rats had less fat mass than H rats. Food intake was higher in C rats compared to H rats. CN rats had lower food intake than C rats. HN rats had the same food intake as H rats. Water intake was higher in H rats compared to C rats, whereas CN and HN rats had the same water intake. Energy intake was highest in H rats compared to C rats. HN rats had the same energy intake as H rats. CN rats had the lowest energy intake (Table 1).

Total abdominal fat was higher in H rats compared to C rats, and HN rats had less abdominal fat than H rats. Epididymal and omental fat pads were the same across all groups. Retroperitoneal fat was higher in H rats compared to C rats, HN rats had less retroperitoneal fat compared to H rats. CN rats were the same as C rats (Table 1).

Plasma total cholesterol concentrations were unchanged among all groups. Plasma triglyceride concentrations were higher in H rats compared to C rats, whereas HN rats were the same as H rats, while CN rats were higher than C rats. Plasma non-esterified fatty acids were the same for C and H rats, but CN and HN rats were higher than their respective controls (Table 1). H rats had higher 120-minute blood glucose

concentrations and area under the curve compared to C rats. CN and HN rats were the same as their respective controls. H rats had higher 120-minute blood glucose concentrations and area under the curve after insulin administration compared to C rats; CN rats were higher than C rats, HN rats were higher than H rats (Table 1).

After eight weeks, systolic blood pressure of H and HN rats were higher than of C and CN rats. Systolic blood pressure in H rats was higher at 16 weeks than in C rats. CN and HN rats were the same as their respective controls. Diastolic stiffness was higher in H rats compared to C rats. CN and HN rats were the same as their respective controls. Left ventricular weights with septum and right ventricular wet weights were the same across all groups. Left ventricles from H rats showed increased infiltration of inflammatory cells and collagen deposition whereas these changes were not seen in left ventricles from C rats. CN and HN rats were the same as their respective controls (Figure 2).

Livers from H rats showed increased fat vacuole size and infiltration of inflammatory cells compared to livers from C rats, while HN rats had decreased fat vacuole size but not inflammatory cells compared to H rats (Figure 2). Plasma activities of ALT and AST were not different between all groups (Table 1).

3.3 Gut structure and microbiome

Histology of ileum and colon did not show any structural abnormalities in the experimental groups demonstrated by normal crypt depth, villi length and goblet cells and lack of inflammatory cell infiltration (Figure 2).

The gut microbiome of the rat was defined as the collective genomes of the microbes in the colon. After quality filtering, there were a total of 720,071 sequences and these were clustered into 1290 zOTUs. The calculated rarefaction curves based on rarefied and unrarefied data as well as Good's coverage of $99.62 \pm 0.1\%$ revealed that the bacterial community was almost fully recovered by the surveying effort. There were no differences for any pairwise comparison of Shannon's diversity between the diets, except for a higher Shannon's diversity in the HN group compared to the C group. There was higher richness in the HN group compared to the C group. An overall effect of treatment was observed on the bacterial community structure for the faecal samples. There were differences between C and H groups indicating an effect of basal feed on the bacterial community structure (Figure 3). The addition of *N. oceanica* (CN and HN) changed the bacterial communities.
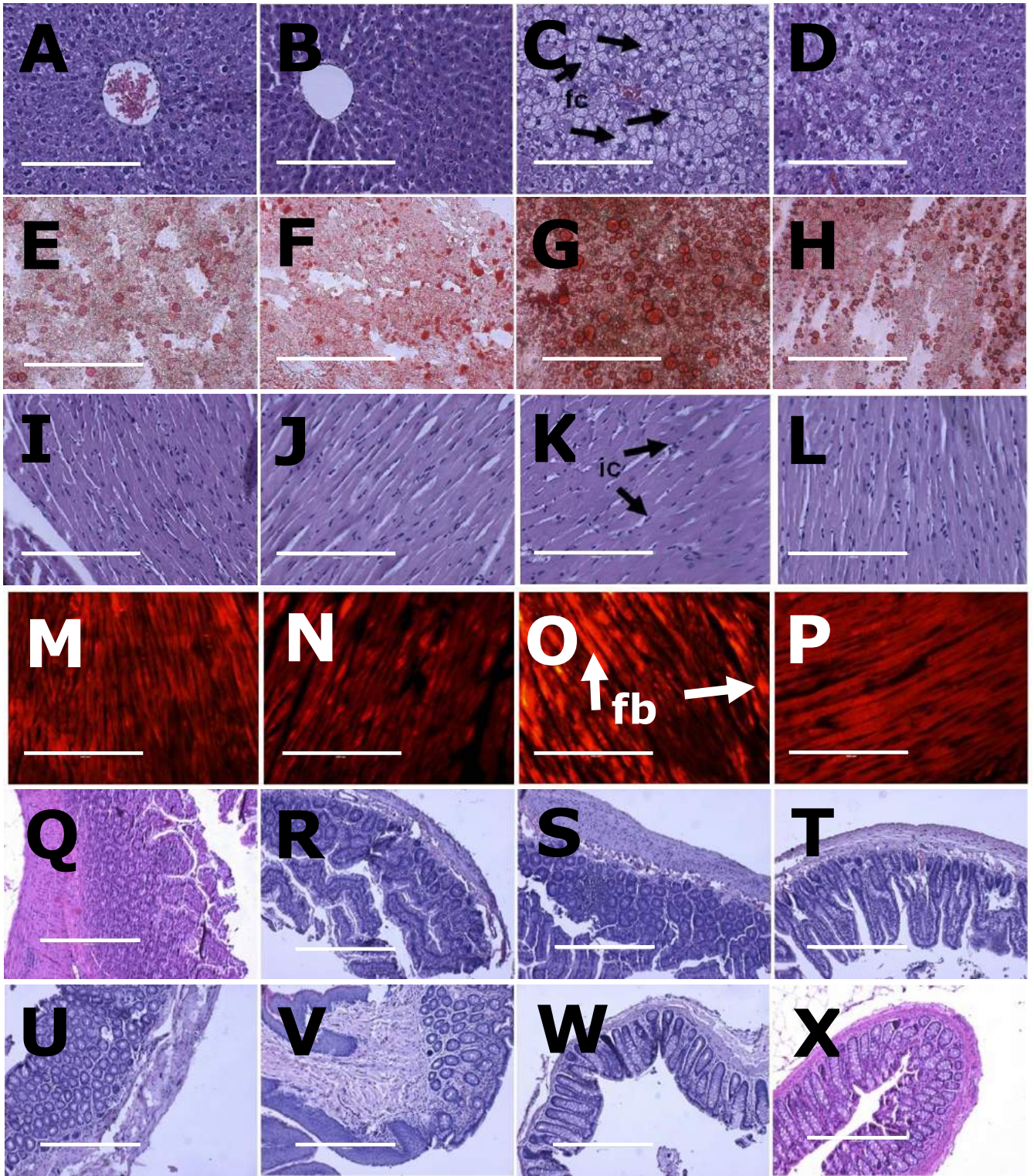
Figure 2. Liver fat using haematoxylin and eosin stain (A-D) and oil red O stain (E-H); heart inflammation (I-L) using haematoxylin and eosin stain; heart fibrosis (M-P) using picrosirius red stain; ileum (Q-T) and colon (U-X) structure using haematoxylin and eosin stain. Each row is in the following order: corn starch diet-fed rats (C), corn starch diet-fed rats supplemented with 5% *N. oceanica* (CN), high-carbohydrate, high-fat diet-fed rats (H) and high-carbohydrate, high-fat diet-fed rats supplemented with 5% *N. oceanica* (HN). Fat cells=fc; inflammatory cells=ic; fibrosis=fb. Scale bar is 200 μm for A-P (20x) and 100 μm for Q-X (10x).
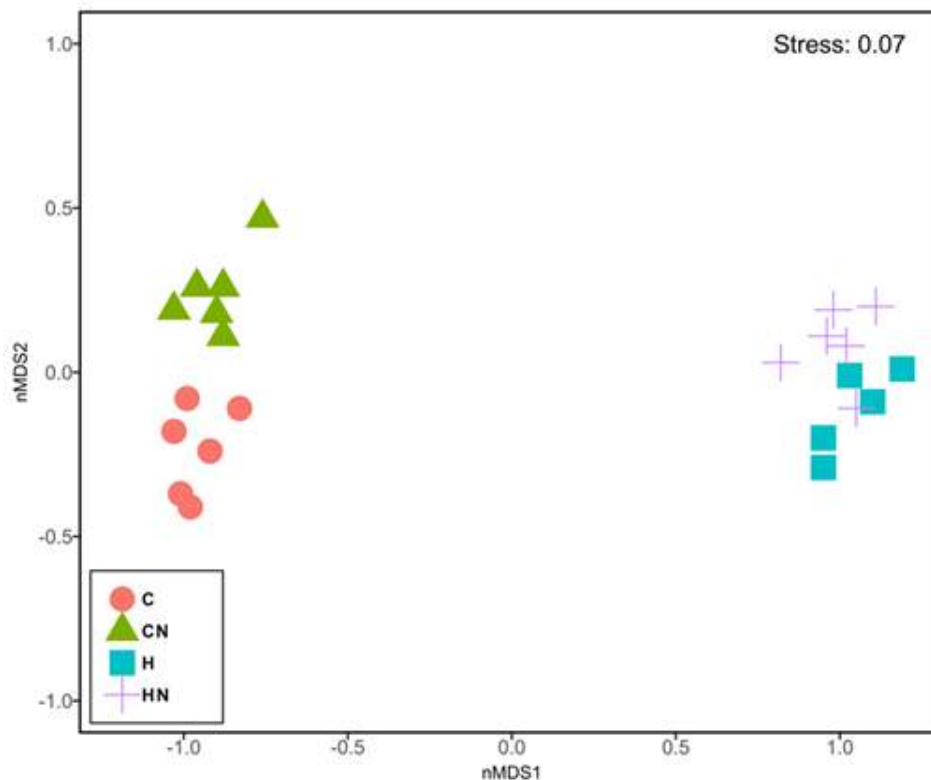

Figure 3. MDS plot of bacterial communities structure of faecal samples from different diets. Corn starch diet-fed rats (C), corn starch diet-fed rats supplemented with 5% *N. oceanica* (CN), high-carbohydrate, high-fat diet-fed rats (H) and high-carbohydrate, high-fat diet-fed rats supplemented with 5% *N. oceanica* (HN).

Table 1. Responses to *Nannochloropsis oceanica*

Variable	C	CN	H	HN	P value		
					Diet	**Treatment**	**D x T**
Physiological variables							
0 wk body weight	337 ± 1	336 ± 1	339 ± 1	337 ± 1	0.1918	0.1918	0.6597
8 wk body weight	366 ± 7[b]	386 ± 2[b]	445 ± 10[a]	430 ± 5[a]	<0.0001	0.6635	0.0043
16 wk body weight	388 ± 10[c]	453 ± 6[b]	547 ± 14[a]	528 ± 10[a]	<0.0001	0.0317	0.0003
8 wk lean/fat mass proportion	6.2 ± 1.6[a]	5.8 ± 0.4[a]	2.1 ± 0.4[b]	2.8 ± 0.3[b]	<0.0001	0.8240	0.4171
16 wk lean/fat mass proportion	3.8 ± 1.0[a]	4.3 ± 0.5[a]	1.5 ± 0.3[b]	2.0 ± 0.2[b]	0.0001	0.3495	1
16 wk lean mass (g)	292 ± 15[b]	328 ± 7[a]	299 ± 13[b]	323 ± 6[a]	0.9167	0.0034	0.5316
16 wk fat mass (g)	75 ± 15[c]	91 ± 12[c]	230 ± 35[a]	172 ± 13[b]	<0.0001	0.2572	0.0504
16 wk bone mineral content (g)	11.6 ± 0.3[b]	12.3 ± 0.4[b]	16.6 ± 1.1[a]	15.0 ± 0.5[a]	<0.0001	0.4610	0.0656
16 wk bone mineral density (g/cm^2)	0.1703 ± 0.0032	0.1659 ± 0.0020	0.1809 ± 0.0041	0.1760 ± 0.0036	0.0052	0.1874	0.9427
16 wk abdominal circumference (cm)	18.7 ± 0.5[c]	21.5 ± 0.2[b]	23.8 ± 0.4[a]	23.4 ± 0.3[a]	<0.0001	0.0013	<0.0001
Body mass index (g/cm^2)	0.61 ± 0.03[c]	0.72 ± 0.01[b]	0.81 ± 0.02[a]	0.77 ± 0.02[a]	<0.0001	0.0936	0.0008
Visceral adiposity (%)	5.2 ± 0.5[b]	5.2 ± 0.3[b]	9.3 ± 1.1[a]	8.4 ± 0.4[a]	<0.0001	0.4174	0.4174
Food intake 0-8 wk (g/day)	43.2 ± 2.2[a]	40.4 ± 0.8[a]	26.6 ± 1.1[b]	26.0 ± 1.2[b]	<0.0001	0.2120	0.4160
Food intake 9-16 wk (g/day)	44.0 ± 1.2[a]	34.9 ± 0.6[b]	23.9 ± 0.9[c]	22.0 ± 1.0[c]	<0.0001	<0.0001	0.0009
Water intake 0-8 wk(g/day)	31.8 ± 1.6	36.8 ± 2.5	32.4 ± 1.4	33.0 ± 1.5	0.4756	0.2156	0.3284
Water intake 9-16 wk(g/day)	21.7 ± 1.4[c]	36.8 ± 2.4[a]	28.8 ± 1.3[b]	37.0 ± 1.6[a]	0.1026	<0.0001	0.1220
Energy intake 0-8 wk (kJ/day)	485 ± 25[b]	454 ± 9[b]	607 ± 19[a]	590 ± 22[a]	<0.0001	0.2429	0.7308
Energy intake 9-16 wk (kJ/day)	470 ± 13[b]	392 ± 7[c]	536 ± 15[a]	533 ± 22[a]	<0.0001	0.0307	0.0444
Cardiovascular variables							
8 wk systolic blood pressure (mmHg)	125 ± 4[b]	126 ± 2[b]	137 ± 3[a]	132 ± 2[a]	0.0019	0.4570	0.2671
16 wk systolic blood pressure (mmHg)	123 ± 2[b]	121 ± 2[b]	139 ± 2[a]	135 ± 3[a]	<0.0001	0.2053	0.6672

Diastolic stiffness (κ, dimensionless)	22.1 ± 0.8^b	22.4 ± 0.9^b	30.1 ± 0.7^a	30.2 ± 0.8^a	<0.0001	0.8059	0.9022
Left ventricle collagen area (%)	6.5 ± 0.6^b	7.2 ± 0.9^b	27.4 ± 2.6^a	26.2 ± 2.1^a	<0.0001	0.8882	0.5946
Organ weights							
Kidneys (mg/mm)	52 ± 3	55 ± 1	58 ± 2	65 ± 2	0.0004	0.0191	0.3308
Spleen (mg/mm)	14.5 ± 0.7^b	19.0 ± 1.4^a	17.0 ± 0.9^a	18.3 ± 1.1^a	0.5056	0.0376	0.2401
Left ventricle + septum (mg/mm)	22.9 ± 1.1	23.8 ± 0.7	25.2 ± 1.1	24.3 ± 0.8	0.1423	1	0.3408
Right ventricle (mg/mm)	4.5 ± 0.7	4.3 ± 0.3	5.3 ± 0.2	5.5 ± 0.4	0.0289	1	0.6503
Liver (mg/mm)	261 ± 11^b	260 ± 6^b	380 ± 12^a	370 ± 12^a	<0.0001	0.6246	0.6887
Retroperitoneal fat (mg/mm)	210 ± 20^c	256 ± 17^c	619 ± 69^a	488 ± 31^b	<0.0001	0.2380	0.0176
Epididymal fat (mg/mm)	89 ± 11^b	90 ± 9^b	199 ± 39^a	182 ± 14^a	<0.0001	0.6651	0.6264
Omental fat (mg/mm)	139 ± 14^b	169 ± 16^b	288 ± 56^a	278 ± 19^a	<0.0001	0.7096	0.4579
Total abdominal fat (mg/mm)	437 ± 42^c	514 ± 37^c	1107 ± 57^a	948 ± 59^b	<0.0001	0.4660	0.0416
Metabolic variables							
Total cholesterol (mmol/L)	1.56 ± 0.08	1.73 ± 0.06	1.57 ± 0.10	1.71 ± 0.06	0.9473	0.0471	0.8429
Triglycerides (mmol/L)	0.43 ± 0.02^c	0.71 ± 0.06^b	1.88 ± 0.31^a	1.58 ± 0.22^a	<0.0001	0.9595	0.1472
Nonesterified fatty acids (mmol/L)	0.38 ± 0.06^b	0.61 ± 0.06^a	0.40 ± 0.03^b	0.71 ± 0.07^a	0.3982	0.0005	0.5721
ALT (U/L)	34 ± 4	31 ± 3	38 ± 2	35 ± 3	0.2494	0.3855	1
AST (U/L)	116 ± 2	101 ± 6	120 ± 12	119 ± 12	0.3025	0.4515	0.5096
Liver inflammatory cells (cells/200μm²)	12 ± 2^b	14 ± 2^b	26 ± 3^a	29 ± 4^a	<0.0001	0.3944	0.8636
Liver fat vacuoles area (μm²)	21.2 ± 1.8^c	22.4 ± 2.3^c	135.1 ± 12.9^a	75.0 ± 4.6^b	<0.0001	0.0004	0.0003
Oral glucose tolerance							
0 wk basal glucose (mmol/L)	2.6 ± 0.1	2.9 ± 0.3	2.6 ± 0.2	2.6 ± 0.2	0.5824	0.5824	0.5824
0 wk AUC (mmol/L/120 minutes)	632 ± 30	598 ± 19	606 ± 19	606 ± 19	0.6957	0.4614	0.4614
8 wk basal glucose (mmol/L)	2.9 ± 0.2	2.8 ± 0.1	3.3 ± 0.1	3.3 ± 0.1	0.0013	0.6968	0.6968
8 wk 120 min glucose (mmol/L)	3.5 ± 0.2^b	3.8 ± 0.2^b	5.0 ± 0.1^a	4.5 ± 0.2^a	<0.0001	0.6481	0.0746

8 wk AUC (mmol/L/120 min)	530 ± 15[b]	558 ± 17[b]	657 ± 22[a]	640 ± 15[a]	<0.0001	0.7701	0.2367
16 wk basal glucose (mmol/L)	2.8 ± 0.2	2.7 ± 0.1	3.3 ± 0.2	3.0 ± 1.1	0.0076	0.1637	0.4812
16 wk 120 min glucose (mmol/L)	3.9 ± 0.2[b]	4.1± 0.2[b]	4.8 ± 0.3[a]	4.8 ± 0.2[a]	0.0020	0.6776	0.6776
16 wk AUC (mmol/L/120 min)	501 ± 21[b]	571 ± 15[a]	617 ± 25[a]	593 ± 16[a]	0.0011	0.2424	0.0207
Insulin tolerance							
8 wk ITT 120 min glucose (mmol/L)	2.9 ± 0.4[b]	3.4 ± 0.4[b]	4.5 ± 0.3[a]	4.3 ± 0.2[a]	0.0017	0.6829	0.3434
8 wk ITT AUC (mmol/L/120 min)	247 ± 58[c]	156 ± 25[d]	408 ± 21[a]	369 ± 22[a]	<0.0001	0.0499	0.0411
16 wk ITT 120 min glucose (mmol/L)	2.7 ± 0.3[b]	3.2 ± 0.3[b]	4.5 ± 0.4[a]	4.1 ± 0.2[a]	0.0001	0.8730	0.1568
16 wk ITT AUC (mmol/L/120 min)	208 ± 37[c]	307 ± 36[b]	404 ± 54[a]	365 ± 16[a]	0.0013	0.4120	0.0649

Values are presented as mean ± SEM, n = 10-12. Means in a row with unlike superscripts (a, b or c) differ, $p < 0.05$. C, corn starch diet-fed rats; CN, corn starch diet-fed rats supplemented with 5% *N. oceanica*; H, high-carbohydrate, high-fat diet-fed rats; HN, high-carbohydrate, high-fat diet-fed rats supplemented with 5% *N. oceanica*. ALT, alanine transaminase; AST, aspartate transaminase; AUC, area under the curve; ITT, insulin tolerance test.

4. Discussion

This study shows that rats supplemented with *N. oceanica* had higher lean mass than rats fed only the basal diet. HN rats had lower whole-body fat including lower abdominal fat and liver fat compared to H rats. However there were no effects of treatment on systolic blood pressure or left ventricular diastolic stiffness.

Microalgae are considered part of a healthy diet as they contain fatty acids, proteins, amino acids, pigments, vitamins and minerals (38, 39). Furthermore, microalgae are a sustainable source of these compounds because their cultivation can be carried out independent of freshwater supply and does not compete with arable land or biodiverse landscapes (40). Microalgal constituents are versatile and have potential applications in energy, pharmaceutical, cosmetics and food industries (41). Microalgae have many uses including as a source of high value oil and protein (42). Further, there are genetic tools that enable gene-specific, mechanistic studies to facilitate the engineering of improved *Nannochloropsis* strains with superior growth or greater bioproduction (43). The optimal conditions to culture omega-3 rich microalgae are a temperature range from 15 to 28°C, pH from 7 to 8, medium light irradiances (50-300 μmol photons m^{-2} s^{-1}) and air enriched with 0-1 % (v/v) CO_2 with nitrogen and phosphorus limitation (44).

As *Nannochloropsis* contains high-value compounds such as lipids and proteins, bio-refineries have been developed to exploit their rapid growth and high oil productivity by various harvesting and extraction techniques (45). There are many techniques to harvest *Nannochloropsis* including solvent extraction, pre-treatments, supercritical fluid extraction and ionic liquid extraction (46). Ionic liquid cholinium arginate was an effective solvent for extracting lipids with only 1.4 ± 0.2% lipids remaining in the *Nannochloropsis* biomass (47). Flocculation also increased *Nannochloropsis* harvesting efficiency (48). There are different ways to increase the valuable components of *Nannochloropsis*. Light wavelength affects microalgal growth due to the presence of photosynthetic pigments which absorb different photons of light at specific wavelengths. Short exposures to UVC (100-280 nm) at a dose of 100 or 250 mJ/cm^2 increased EPA content; 100 mJ/cm^2 for 24 hours doubled EPA content to ~7% (17). Further, *Nannochloropsis* production can be increased under certain light intensities and wavelengths as demonstrated by an experiment with LEDs (49). Similar biomass and fatty acid yields were obtained at high-light intensity (150 μmol m^{-2} s^{-1}) LEDs on day 7 and low-light intensity (50 μmol m^{-2} s^{-1}) LEDs on day 11

during cultivation. However, the power efficiencies of biomass and fatty acid (specifically EPA) production were higher for low-light intensity. Furthermore, red LEDs resulted in the highest biomass and fatty acid power efficiency (49).

The effects of *Nannochloropsis* on changes in metabolic syndromes have been reported. In rats, streptozocin was used to produce acute pancreatic β−cell damage and induce hyperglycaemia. The marine-water microalga, *N. oculata*, and its extract minimised the pancreatic tissue damage and maintained the integrity of the genomic DNA (50). *N. oculata* is a good source of $n−3$ PUFA, specifically EPA. Intervention with 10 mL/kg body weight for 14 days had no effect on body weight which is similar to the current study (51). Studies show that $n−3$ PUFA, especially α-linolenic acid, EPA and DHA reduce cardiovascular disease risk factors (10). Other studies using the same metabolic syndrome model as the current study showed EPA and DHA (52) as well as oleic acid present in olive leaves (53) improved cardiovascular and hepatic parameters.

There are other uses for microalgae due to the high concentrations of fatty acids, with *Nannochloropsis* species used for production of biofuels such as biodiesel (54). The advantages of biodiesel from microalgae as an alternative energy source for the future are numerous (55). When compared to other sources such as animal fat, oleaginous grain crops and oil palm, microalgae (per unit of land) have a higher growth rate and oil productivity of microalgae than other biofuel crops (16). Further, algae grow in a wide range of environments such as fresh, brackish and saline waters (16). Thus, a portion of *Nannochloropsis* grown for other uses could be diverted for the development of functional food products.

Nannochloropsis components such as whole biomass, pigments, long-chain PUFA, triglycerides, alkanes and alkenes have many biotechnological applications including aquaculture, fish food, livestock feeds, health foods, biofuels and wastewater treatment (56). Furthermore, 1,950 proteins from *N. gaditana* have been identified, some with a potential role in different agri-food and biomedical applications (57). Therefore, mapping the *N. gaditana* proteome may help to understand processes and be key to the expansion of biotechnological applications of this microalga (57). *N. oceanica*-derived defatted meal can be used as a sustainable source of EPA to allow accumulation by fish as fish oils (58).

It has been suggested that *Nannochloropsis* can be added to food, such as bread, pasta and protein bars to create highly nutritious functional foods (46). The addition of *N. gaditana* to bread changed the colour to green-yellow crust and crumb, suggesting an increased browning. The textural parameters of the bread such as hardness, chewiness and resilience were unchanged (59). Microalgae-supplemented bread would address the general deficiency of $n-3$ PUFA and minerals, such as zinc, in the human population (46), although the change in colour may decrease consumer acceptance. Marine natural pigments as potential sources for antioxidant, anticancer, antiangiogenic and anti-obesity therapeutic applications have been reviewed (60). Microalgae such as *Porphyridium cruentum, Isochrysis galbana, Phaeodactylum tricornutum, Tetraselmis suecica* and *N. gaditana* have species-specifc carotenoid profiles with a prevalence of xanthophylls over carotenes (61). β-carotene was the only carotenoid common to all species. Violaxanthin was reported as the prevalent carotenoid (336.7 mg 100 g^{-1} dw) in *N. gaditana* (61).

The validated dietary model of human metabolic syndrome in this study mimics the obesity and cardiovascular changes in rats (23). Further, interventions with seaweeds and other microalgae reversed these changes (62, 63). This study was limited because the cell wall of *N. oceanica* was not sheared and thus nutrients and bioactives were unlikely to be bioavailable to rats. Cell wall thickness in *Nannochloropsis* species varies from 63 to 119 nm due to the distinct genetic traits in each strain with *N. oeanica* having one of the thickest cell walls (64).

5. Conclusions

This study was limited because the cell wall of *N. oceanica* was not sheared and thus nutrients and bioactives were probably less bioavailable to rats. Hence, the hypothesised responses in rats with diet-induced metabolic syndrome did not occur to their full effect.

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CHAPTER 7. CRCUMIN NANOPARTICLES IN DIET-INDUCED METABOLIC SYNDROME

Oral administration of drugs for systemic disease assumes sufficient bioavailability for the drug to get to the site of action in the body. There are many physicochemical processes to improve oral bioavailability, with one common one being the incorporation of compounds with low bioavailability in nanoparticles. This chapter investigated the effectiveness of nanoparticles to improve the absorption of the poorly-absorbed natural product, curcumin, in a reversal model of diet-induced metabolic syndrome in rats. Curcumin has been used for thousands of years for purported health benefits but it is limited by its low bioavailability of around 1%. This chapter follows on from the previous chapters where seaweeds were studied to investigate the health benefits of polysaccharides. Seaweed components such as carotenoids have known health benefits but have low oral bioavailability, similar to curcumin. This study aimed to determine if high-dose unformulated curcumin attenuates symptoms of metabolic syndrome and to compare the effects to low-dose curcumin nanoparticles. Nanoparticles are an effective drug delivery system because they target specific tissues, thereby reducing dose and unwanted side effects. A possible future application of seaweed-derived polysaccharides, such as alginates and other biopolymers, is to encapulsate high-value seaweed-derived carotenoids, such as fucoxanthin and astaxanthin, in order to increase their bioavailability.

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Article

Low-Dose Curcumin Nanoparticles Normalise Blood Pressure in Male Wistar Rats with Diet-Induced Metabolic Syndrome

Ryan du Preez [1,2], **Jessica Pahl [1,2]**, **Meenakshi Arora [3]**, **M. N. V. Ravi Kumar [3]**, **Lindsay Brown [1,2]** and **Sunil K. Panchal [1,*]**

[1] Functional Foods Research Group, University of Southern Queensland, Toowoomba, QLD 4350, Australia
[2] School of Health and Wellbeing, University of Southern Queensland, Toowoomba, QLD 4350, Australia
[3] Texas A&M Rangel College of Pharmacy, Texas A&M University–College Station, TX 77843, USA
[*] Correspondence: Sunil.Panchal@usq.edu.au; Tel.: +61-7-4631-1012

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Abstract: Nanoparticle formulations improve bioavailability and so may allow low-dose formulations of food-derived compounds such as curcumin to attenuate chronic systemic disease despite intrinsically low oral bioavailability. The current study induced metabolic syndrome in male Wistar rats aged eight–nine weeks using a high-carbohydrate, high-fat diet (H) with corn starch diet (C) as control. Using a reversal protocol, rats were given curcumin as either nanoparticles encapsulated in poly(lactic–co–glycolic acid) (5 mg/kg/day, HCNP) or as an unformulated low dose or high-dose suspension in water (low-dose, 5 mg/kg/day, HC5; high-dose, 100 mg/kg/day, HC100) or blank nanoparticles (HBNP) for the final eight weeks of the 16 week study. We analysed cardiovascular parameters including systolic blood pressure and left ventricular diastolic stiffness along with histopathology, liver parameters including plasma liver enzymes, histopathology and metabolic parameters, including glucose tolerance, blood lipid profile and body composition, and plasma curcumin concentrations. HC100 and HCNP but not HBNP normalised systolic blood pressure (C = 120 ± 4; H = 143 ± 5; HBNP = 141 ± 3; HC5 = 143 ± 4; HC100 = 126 ± 4; HCNP = 128 ± 4 mmHg), left ventricular diastolic stiffness and liver fat deposition. No other improvements were induced in HC100 or HCNP or other intervention groups (HC5 and HBNP). We conclude that 5 mg/kg/day curcumin nanoparticles in H rats showed similar improvements in cardiovascular function as 100 mg/kg/day unformulated curcumin correlating with similar plasma curcumin concentrations.

Keywords: metabolic syndrome; curcumin; nanoparticles; hypertension; obesity; oral bioavailability

1. Introduction

Curcumin is the major active constituent of turmeric, isolated from the rhizomes of *Curcuma longa*. Turmeric is commonly used as a spice and food-colouring agent while curcumin has been proposed for the management of a wide range of diseases including metabolic syndrome, arthritis, anxiety and hyperlipidaemia [1], as well as ageing-associated diseases including cardiovascular disease and chronic inflammation [2]. The broad-spectrum activity of curcumin has been attributed to its ability to modulate many transcription factors, cytokines, growth factors and enzymes [3]. However, the therapeutic usefulness of curcumin has been strongly questioned because of its interference with many assays and producing misleading and false results leading to the description of curcumin as an unstable, reactive and non-bioavailable compound, and to its classification as a pan-assay interference compound and an invalid metabolic panacea candidate [4]. This review concluded that curcumin does not warrant further investigation as a therapeutic agent [4]. This is in marked contrast with the many

clinical trials that have shown safety, tolerability and effectiveness of curcumin against chronic diseases in humans [1–3,5].

Turmeric contains hundreds of potentially bioactive compounds with curcumin present at 0.4%–7.2% of dry weight [6]. However, curcumin has very low oral bioavailability in rats, due to its hydrophobic nature, rapid metabolism and systemic elimination. A comparison of intravenous and oral administration routes showed that 10 mg/kg intravenous curcumin produced an area under the curve of 7.2 ± 1.2 μg/mL × minutes, in contrast to 3.6 ± 0.6 μg/mL × minutes following 500 mg/kg oral curcumin, thus showing ~1% oral bioavailability [7]. Alternative delivery methods have focussed on increasing bioavailability and absorption, including adjuvants, liposomes, micronisation and nanoparticles, aiming to produce health benefits using a lower dose of curcumin [8]. Adjuvants such as piperine inhibited hepatic and intestinal glucuronide conjugation to increase curcumin bioavailability [9]. Liposomes should be an effective carrier to enhance the stability, bioavailability and targeting property of curcumin [10]. A γ-cyclodextrin curcumin formulation increased relative bioavailability of total curcuminoids in healthy humans by 39-fold compared to standard curcumin [11]. Nanoparticle preparations of curcumin encapsulated in poly (lactic–co–glycolic acid) (PLGA) increased bioavailability by ~9-fold more than co-administration with piperine which was 3-fold more than unformulated curcumin [12]. Curcumin nanoformulations increased solubilisation of curcumin and protected curcumin against inactivation by hydrolysis [13].

Metabolic syndrome is defined as a clustering of risk factors associated with increased risk of cardiovascular disease and diabetes, including abdominal obesity, hypertension, impaired glucose tolerance, insulin resistance, decreased high-density lipoprotein-cholesterol and elevated triglycerides [14]. Among US adults, metabolic syndrome has increased by more than 35% in the last two decades [15]. The use of functional foods and nutraceuticals could produce a reliable decrease in metabolic syndrome with decreased comorbidities. This outcome would be superior to current drug treatments [16].

In the current study, metabolic syndrome was induced in male rats by feeding a diet with increased simple sugars such as fructose and sucrose together with increased saturated and *trans* fats. These rats were treated with PLGA nanoparticle-encapsulated curcumin using a reversal protocol since these nanoparticles showed improved biocompatibility, biodegradability and capacity to prevent premature degradation of curcumin without toxicity during chronic dosing [17,18]. PLGA nanoparticle encapsulation of curcumin improved oral bioavailability, delayed cataract progression and prevented *ß*-cell death [12,17,19]; however, this is the first study investigating oral low-dose curcumin PLGA-nanoparticles in a rat model of human metabolic syndrome. PLGA has been used in products that are in human use and is a widely used polymer for drug delivery applications [20].

Curcumin was delivered orally to rats as a suspension at 5 or 100 mg/kg/day to provide both an equal dose and an approximately equal predicted absorption of curcumin at 100 mg/kg/day with PLGA nanoparticles at 5 mg/kg/day. After treatment with curcumin, the structure and function of the cardiovascular system and liver were examined. Cardiovascular structure and function were studied in isolated Langendorff heart preparations and by measurement of systolic blood pressure together with histopathology. Liver structure and function were measured by plasma biochemical analysis and histopathology. Metabolic parameters related to obesity and glucose tolerance were also evaluated. Further, we characterised the composition of the gut microbiome after curcumin treatment, since obesity-induced changes in the gut microbiome in metabolic disorders, including cardiovascular disease, may be reversed by functional foods, nutraceuticals and herbal medicines [21,22]. We hypothesised that rats given low-dose curcumin nanoparticles (5 mg/kg/day) will have similar chronic physiological responses as rats given the high-dose curcumin suspension (100 mg/kg/day) and much greater than rats given the low-dose curcumin suspension (5 mg/kg/day), showing that a lower curcumin dose in nanoparticles is effective against signs of metabolic syndrome.

2. Materials and Methods

2.1. Curcumin Suspension and Nanoparticles

Curcumin was obtained from Acros Organics (Morris Plains, NJ, USA) as a mixture of curcumin (>98%) with demethoxycurcumin and bisdemethoxycurcumin (both <1%). Curcumin aqueous suspensions were prepared as 5 and 100 mg/mL. PLGA ((50:50) Resomer R503H; MW 35-40kDa) was used to prepare curcumin nanoparticles (CNP) by single emulsion method resulting in particles with a hydrodynamic diameter of 315–320 nm [17,19]. Blank nanoparticles (BNP) without curcumin were used as the vehicle control and had a hydrodynamic diameter of 310 nm. Both CNP and BNP had surface charge measured as zeta potential of –29 mV at pH 5.5.

2.2. Rats and Diets

All experimental protocols were approved by the Animal Ethics Committee of the University of Southern Queensland (approval number 15REA008) under the guidelines of the National Health and Medical Research Council of Australia. Male Wistar rats (8–9 weeks old; 338 ± 1 g, *n* = 120) were obtained from the Animal Resource Centre, Murdoch, WA, Australia. Rats were individually housed in a temperature-controlled (21 ± 2 °C), 12-hour light/dark conditions with free access to food and water. Rats were randomly distributed into ten groups, each with 12 rats. For the first eight weeks, half the rats received a corn starch (C) diet and the other half received a high-carbohydrate, high-fat (H) diet [23]. Two groups (C and H rats) received the diet uninterrupted throughout the remaining eight weeks of the sixteen-week study. The remaining eight groups were administered curcumin suspension (5 or 100 mg/kg/day; CC5, CC100, HC5 or H100) or blank or curcumin nanoparticles (CBNP, CCNP, HBNP or HCNP) by once-daily oral gavage together with C or H diet for the remaining eight weeks. The energy densities were 11.23 kJ/g for the C food, 17.83 kJ/g for the H food with an additional 3.85 kJ/mL from the fructose in the drinking water of H rats.

2.3. Measurements Before Euthanasia

Systolic blood pressure was measured under light sedation with Zoletil (10 mg/kg tiletamine, 10 mg/kg zolazepam, i.p; Virbac, Peakhurst, NSW, Australia) [23]. Measurements were performed using an MLT1010 Piezo-Electric Pulse Transducer (ADInstruments, Bella Vista, NSW, Australia) and an inflatable tail-cuff connected to an MLT844 Physiological Pressure Transducer (ADInstruments) connected to a PowerLab data acquisition unit (ADInstruments).

Oral glucose tolerance tests were performed on rats after overnight (12-hour) food deprivation. Fructose-supplemented drinking water in H, HC5, HC100, HBNP and HCNP rats was replaced with normal drinking water. Basal blood glucose concentrations were determined in tail vein blood using Medisense Precision Q.I.D. glucometer (Abbott Laboratories, Bedford, MA, USA) and glucose test strips (Freestyle Optium Blood Glucose Test Strips, Abbott Diabetes Care Ltd, Milperra NSW, Australia). Rats were then given 2 g/kg body weight of glucose as a 40% (w/v) aqueous solution by oral gavage. Following this, blood glucose concentrations were measured at 30, 60, 90 and 120 min following glucose administration [23].

Dual-energy X-ray absorptiometry was performed on all rats after 16 weeks of feeding using a Norland XR46 DXA instrument (Norland Corp., Fort Atkinson, WI, USA). Rats were sedated with Zoletil (10 mg/kg tiletamine, 10 mg/kg zolazepam; i.p). Scans were analysed using the manufacturer's recommended software for use in laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1; Norland Corp.) [23].

Indirect calorimetry was used to measure oxygen consumption and carbon dioxide production using a 4-chamber Oxymax system (Columbus Instruments, Columbus, OH, USA) with one rat per chamber. Rats had free access to food and water during the measurement. Oxygen consumption (V_{O2}) and carbon dioxide production (V_{CO2}) were measured individually from each chamber. The respiratory exchange ratio (RER = V_{CO2}/V_{O2}) was calculated by Oxymax software (v. 4.86). The oxidation of

carbohydrates produces an RER of 1.00, whereas fatty acid oxidation results in an RER of about 0.70. Energy expenditure (heat) was calculated by assessment of the exchange of oxygen for carbon dioxide that occurs during the metabolic processing of food [24].

2.4. Measurements After Euthanasia

Rats were euthanased via intraperitoneal injection of Lethabarb (pentobarbitone sodium, 100 mg/kg; Virbac). Once euthanasia was induced in rats, heparin was administered (~200 IU) into the right femoral vein. The abdomen was then opened and blood (~6 mL) was withdrawn from the abdominal aorta, collected into heparinised tubes and centrifuged at $5000 \times g$ for 10 min. Plasma from each rat was stored at $-20\,^\circ$C before analysis. Hearts were removed for use in the isolated Langendorff heart preparation. Hearts were perfused with a modified Krebs-Henseleit bicarbonate buffer [23]. Buffer was bubbled with 95% O_2–5% CO_2 and maintained at 35 $^\circ$C. Isovolumetric ventricular function was measured by inserting a latex balloon catheter into the left ventricle connected to a Capto SP844 MLT844 physiological pressure transducer and Chart software on a MacLab system. All left ventricular end-diastolic pressure values were measured during pacing of the heart at 250 beats/min using an electrical stimulator. End-diastolic pressures were obtained from 0 to 30 mmHg for calculation of diastolic stiffness constant (κ, dimensionless) [23].

After completing the Langendorff heart preparation, hearts were separated into right ventricle and left ventricle with septum for weighing. Livers and abdominal fat pads (retroperitoneal, epididymal and omental) were isolated and weighed. These organ weights were normalised relative to the tibial length at the time of their removal (in mg of tissue/mm of tibial length) [23].

Two rats from each group were exclusively used for histological analysis. Tissues were also collected from two other rats in each group. Approximately 5–7 min after euthanasia, heart, liver and intestinal tissues were collected and fixed in 10% neutral buffered formalin for 3 days. The samples were then dehydrated and embedded in paraffin wax. Two slides were prepared for each heart and liver specimen and two random, non-overlapping fields per slide were taken to avoid biased analysis. Thin sections (5 μm) of the samples were cut and stained with haematoxylin and eosin for determination of inflammatory cell infiltration (20×) and presence of liver fat vacuoles (40×) or picrosirius red stain for collagen deposition. EVOS FL Colour Imaging System (v1.4 (Rev 26059); Advanced Microscopy Group, Bothell, WA, USA) was used to capture images to determine the extent of collagen deposition in selected tissue sections [23]. NIH ImageJ software was used to quantify collagen deposition in heart sections and to count inflammatory cells in liver sections.

Plasma samples collected during terminal experiments were used to test for enzyme activities and plasma concentrations of biochemical markers. Plasma activities of alanine transaminase (ALT) and aspartate transaminase (AST) and plasma concentrations of total cholesterol, triglycerides and non-esterified fatty acids were determined using kits and controls [23].

Immediately following euthanasia and organ removal, two or three faecal pellets were collected from the colon of rats and stored at $-80\,^\circ$C in nuclease-free tubes. DNA extraction and diversity profiling were performed by the Australian Genome Research Facility, Brisbane, QLD, Australia. The V3-V4 region of the 16S rRNA gene was selected for amplification. The primers used were F341 (50-CCTAYGGGRBGCASCAG-30) and R806 (50-GGACTACNNGGGTATCTAAT-30). PCR amplicons were generated using AmpliTaq Gold 360 mastermix (Life Technologies, Scoresby, VIC, Australia) for the primary PCR. Detailed description for the analysis of diversity profiling is available in our previous study [25].

Plasma concentrations of curcumin were measured by LC-MS/MS. Stock solutions at a concentration of 1.0 mg/mL were prepared by separately dissolving 1 mg of curcumin, curcumin glucuronide and salbutamol (internal standard) in 1 mL of acetonitrile. Standard working solutions were then prepared by dilution of curcumin and curcumin glucuronide stock solutions with acetonitrile to obtain working solutions with concentrations of 1, 2.5, 5, 10, 25, 50, 100 and 250 μg/mL. Calibration standards were prepared by spiking 30 μL of blank rat serum with a freshly prepared working solution

at concentrations of 1, 2.5, 5, 10, 25, 50, 100 and 250 μg/mL and extracting with 10 ng/mL salbutamol in 100% acetonitrile to achieve standards with concentrations of 0.033, 0.0833, 0.166, 0.33, 0.833, 1.66, 3.33 and 8.33 μg/mL. For sample preparation, 30 μL of rat plasma samples were placed in a 1.5 mL Eppendorf tube, 120 μL of ice-cold 10 ng/mL salbutamol in 100% acetonitrile was added and the mixture was vortexed. Samples were sonicated in a water bath for 1 min and then centrifuged at 15,000 rpm for 5 min. After centrifugation, 200 μL of the supernatant was transferred to a clean 1.5 mL Eppendorf tube. 50 μL of the supernatant was then transferred to a 2 mL glass vial with a glass insert from where 10 μL was injected into the LC–MS/MS system. The target compounds in samples were detected and quantified on a triple quadrupole mass spectrometer (Quantiva, Thermo Fisher Scientific, Waltham, MA, USA) coupled to a binary pump HPLC (UltiMate 3000, Thermo Fisher Scientific). MS parameters were optimised for the target compound under direct infusion at 5 μL/min to identify the SRM transitions (precursor/product fragment ion pair) with the highest intensity in positive mode as 369.4–177 m/z for curcumin, 545.2–369 m/z for curcumin glucuronide and 240.2–148.2 m/z for salbutamol. Samples were maintained at 4 °C on an autosampler before injection. The injection volume was 10 μL. Chromatographic separation was achieved on a Hypersil Gold 5 μm 50 × 3 mm column (Thermo Scientific) maintained at 30 °C using a solvent gradient method. Solvent A was water with 0.1% formic acid. Solvent B was acetonitrile with 0.1% formic acid. The gradient method used was 0–4 min (20% B to 80% B), 4–4.1 min (80% B to 95% B), 4.1–6 min (95% B), 6–6.5 min (95% B to 20% B) and 6.5–8 min (20% B). The flow rate was 0.5 mL/min. Sample acquisition and analysis were performed with TraceFinder 3.3 (Thermo Scientific).

2.5. Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM). Results were tested for variance using Bartlett's test and variables that were not normally distributed were transformed (using log10 function) prior to statistical analyses. Data from the ten treatment groups were tested by two-way analysis of variance. When the interactions and/or the main effects were significant, means were compared using the Newman-Keuls multiple comparison post hoc test. Where transformations did not result in normality or constant variance, a Kruskal-Wallis non-parametric test was performed. A *p* value of <0.05 was considered as statistically significant. All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Body Parameters and Dietary Intakes

Rats fed a high-carbohydrate, high-fat diet as simple sugars and both saturated and *trans* fats (H rats) developed symptoms characteristic of human metabolic syndrome, including abdominal obesity (shown as increases in body weight, fat mass and abdominal fat pads), hypertension (shown as increases in systolic blood pressure), dyslipidaemia (shown as increases in plasma triglycerides and non-esterified fatty acids) and impaired glucose tolerance (shown as increases in 120-min blood glucose concentrations and area under the curve) compared to rats fed the corn starch diet (C rats) (Table 1). Body weights were unchanged with the interventions compared to their respective controls (Table 1). Lean mass was unchanged in all groups (Table 1). Fat mass was unchanged between C groups or between H, HC5, HC100 and HCNP rats (Table 1). Food intake was higher in C rats compared to H rats. CC5 and CC100 rats had similar food intake to C rats, whereas CCNP had higher food intake than CBNP rats, which were higher than C, CC5 and CC100 rats (Table 1). With lower body weight gain, all C diet-fed groups had lower feed efficiency compared to H diet-fed groups (Table 1). Plasma curcumin concentrations measured at the end of the protocol were unchanged between CC100 and CCNP or HC100 and HCNP groups, suggesting improved oral bioavailability with nanoparticle delivery. On the other hand, plasma curcumin concentrations in CC100 was higher than in HC100 rats, but not in CCNP and HCNP rats. Curcumin was not detected in the plasma from HC5 rats (Table 1).

Table 1. Responses to curcumin and curcumin nanoparticles.

Variables	C	CC5	CC100	CCNP	CBNP	H	HC5	HC100	HCNP	HBNP
Initial body weight, g	337 ± 1	338 ± 1	338 ± 1	338 ± 1	339 ± 1	338 ± 1	339 ± 1	337 ± 1	337 ± 1	339 ± 1
Final body weight, g	393 ± 7 [c]	388 ± 8 [c]	380 ± 8 [c]	403 ± 6 [c]	380 ± 7 [c]	514 ± 10 [ab]	492 ± 9 [b]	498 ± 9 [b]	490 ± 6 [b]	538 ± 16 [a]
Body weight gain (weeks 9–16), %	9.7 ± 1.0 [b]	9.0 ± 1.4 [b]	9.6 ± 0.9 [b]	11.0 ± 1.1 [b]	8.9 ± 1.0 [b]	23.4 ± 1.0 [a]	19.4 ± 1.3 [a]	19.8 ± 1.5 [a]	21.6 ± 1.3 [a]	21.7 ± 1.7 [a]
Final lean mass, g	295 ± 5	289 ± 7	291 ± 8	288 ± 7	286 ± 8	320 ± 8	289 ± 8	291 ± 7	309 ± 11	303 ± 6
Final fat mass, g	85 ± 7 [c]	70 ± 7 [c]	66 ± 6 [c]	104 ± 4 [c]	77 ± 7 [c]	184 ± 10 [b]	185 ± 14 [b]	202 ± 13 [b]	169 ± 9 [b]	233 ± 14 [a]
Water intake (weeks 9–16), mL/day	31.9 ± 2.8	24.9 ± 1.2	28.5 ± 3.2	26.9 ± 1.6	30.6 ± 1.8	27.8 ± 1.1	31.6 ± 1.9	26.2 ± 1.2	31.3 ± 0.8	28.5 ± 1.5
Food intake (weeks 9–16), g/day	39.1 ± 1.3 [c]	39.1 ± 2.2 [c]	38.5 ± 1.5 [c]	49.7 ± 0.7 [a]	44.8 ± 2.1 [b]	27.9 ± 0.9 [ef]	23.7 ± 0.7 [f]	25.0 ± 0.9 [f]	33.6 ± 1.3 [d]	31.3 ± 1.9 [de]
Energy intake (weeks 9–16), kJ/day	439 ± 14 [c]	432 ± 17 [c]	446 ± 15 [c]	559 ± 8 [b]	512 ± 27 [b]	588 ± 15 [b]	561 ± 15 [b]	539 ± 14 [b]	714 ± 23 [a]	661 ± 37 [a]
Feed efficiency (weeks 9–16), g/kJ	0.07 ± 0.01 [b]	0.07 ± 0.01 [b]	0.07 ± 0.01 [b]	0.07 ± 0.01 [b]	0.06 ± 0.01 [b]	0.15 ± 0.01 [a]	0.13 ± 0.01 [a]	0.15 ± 0.01 [a]	0.12 ± 0.01 [a]	0.15 ± 0.01 [a]
Retroperitoneal fat, mg/mm	242 ± 13 [d]	208 ± 15 [d]	211 ± 10 [d]	229 ± 13 [d]	179 ± 17 [d]	481 ± 27 [b]	337 ± 37 [c]	436 ± 22 [b]	467 ± 22 [b]	581 ± 39 [a]
Epididymal fat, mg/mm	76 ± 8 [cd]	79 ± 8 [cd]	68 ± 8 [d]	68 ± 8 [d]	77 ± 6 [cd]	171 ± 13 [b]	171 ± 17 [b]	143 ± 13 [b]	126 ± 11 [bc]	244 ± 29 [a]
Omental fat, mg/mm	137 ± 10 [c]	137 ± 12 [c]	123 ± 7 [c]	134 ± 7 [c]	161 ± 20 [c]	244 ± 14 [ab]	224 ± 11 [b]	219 ± 11 [b]	219 ± 13 [b]	278 ± 19 [a]
Total abdominal fat, mg/mm	455 ± 25 [c]	423 ± 34 [c]	402 ± 23 [c]	432 ± 24 [c]	417 ± 38 [c]	895 ± 44 [b]	827 ± 51 [b]	797 ± 39 [b]	813 ± 38 [b]	1103 ± 70 [a]
Left ventricle + septum weight, mg/mm	23.8 ± 1.4	21.9 ± 0.7	21.0 ± 0.8	22.7 ± 1.1	19.9 ± 0.8	23.4 ± 0.8	22.5 ± 0.5	23.4 ± 1.0	23.4 ± 0.8	24.2 ± 1.0
Right ventricular weight, mg/mm	5.1 ± 0.3 [abc]	4.1 ± 0.3 [cd]	4.7 ± 0.3 [bc]	3.4 ± 0.3 [d]	4.1 ± 0.5 [cd]	5.7 ± 0.2 [ab]	4.8 ± 0.3 [abc]	4.9 ± 0.2 [abc]	4.0 ± 0.2 [cd]	5.9 ± 0.1 [a]
Metabolic variables										
Heat, kcal	3.87 ± 0.08 [ab]	3.86 ± 0.09 [ab]	3.48 ± 0.41 [b]	2.70 ± 0.18 [c]	3.39 ± 0.26 [b]	4.34 ± 0.09 [a]	4.22 ± 0.12 [a]	4.28 ± 0.10 [a]	3.26 ± 0.21 [b]	3.45 ± 0.13 [b]
RER	1.03 ± 0.03 [ab]	1.03 ± 0.10 [ab]	1.04 ± 0.02 [a]	1.03 ± 0.02 [ab]	1.02 ± 0.02 [ab]	0.91 ± 0.01 [ab]	0.92 ± 0.01 [ab]	0.90 ± 0.01 [ab]	0.87 ± 0.02 [b]	0.92 ± 0.01 [ab]
Plasma triglycerides, mmol/L	0.53 ± 0.06 [b]	0.49 ± 0.07 [b]	0.41 ± 0.03 [b]	0.59 ± 0.06 [b]	0.83 ± 0.15 [b]	1.71 ± 0.45 [a]	1.77 ± 0.56 [a]	1.53 ± 0.06 [a]	1.52 ± 0.15 [a]	1.64 ± 0.21 [a]
Plasma total cholesterol, mmol/L	1.64 ± 0.08 [ab]	1.45 ± 0.06 [b]	1.44 ± 0.06 [b]	1.60 ± 0.06 [ab]	1.73 ± 0.13 [ab]	1.53 ± 0.08 [b]	1.71 ± 0.10 [ab]	1.49 ± 0.09 [b]	1.74 ± 0.05 [ab]	1.93 ± 0.13 [a]
Plasma non-esterified fatty acids, mmol/L	1.40 ± 0.20 [cd]	1.28 ± 0.09 [cd]	0.96 ± 0.08 [d]	1.58 ± 0.16 [cd]	2.42 ± 0.33 [bc]	3.30 ± 0.40 [ab]	2.64 ± 0.68 [bc]	4.03 ± 0.36 [a]	3.73 ± 0.18 [ab]	4.50 ± 0.63 [a]
Basal blood glucose, mmol/L	3.2 ± 0.1 [c]	3.6 ± 0.1 [bc]	3.2 ± 0.1 [c]	3.9 ± 0.1 [abc]	3.3 ± 0.2 [c]	3.4 ± 0.2 [bc]	3.9 ± 0.2 [abc]	3.9 ± 0.4 [abc]	4.5 ± 0.2 [a]	4.2 ± 0.2 [ab]
120-min blood glucose, mmol/L	4.6 ± 0.4 [bcd]	4.2 ± 0.1 [cd]	3.7 ± 0.1 [d]	4.5 ± 0.2 [bcd]	3.6 ± 0.2 [d]	6.0 ± 0.6 [a]	5.1 ± 0.2 [abc]	5.4 ± 0.3 [abc]	4.3 ± 0.2 [cd]	5.6 ± 0.4 [ab]
Blood glucose area under the curve, mmol/L×min	665 ± 8 [ab]	591 ± 11 [bc]	561 ± 18 [c]	664 ± 14 [ab]	560 ± 22 [c]	739 ± 35 [a]	695 ± 15 [a]	673 ± 28 [ab]	705 ± 14 [a]	692 ± 42 [a]
Plasma ALT activity, U/L	36 ± 2	41 ± 4	40 ± 5	32 ± 3	32 ± 3	42 ± 2	47 ± 5	40 ± 3	41 ± 3	42 ± 4
Plasma AST activity, U/L	88 ± 2	103 ± 6	90 ± 5	83 ± 3	87 ± 4	95 ± 2	105 ± 10	99 ± 9	88 ± 4	97 ± 10
Plasma curcumin concentrations, ng/ml	-	97.4 ± 18.0 [b]	337.7 ± 84.7 [a]	199.7 ± 45.3 [b]	-	-	0.0 ± 0.0 [c]	146.0 ± 21.2 [b]	110.7 ± 17.8 [b]	-
Liver inflammatory cells (cells/200μm²)	5 ± 1 [c]	6 ± 2 [c]	5 ± 1 [c]	5 ± 2 [c]	5 ± 2 [c]	23 ± 2 [a]	15 ± 3 [b]	16 ± 2 [b]	15 ± 1 [b]	16 ± 1 [b]
Cardiovascular variables										
16 week systolic blood pressure, mmHg	120 ± 4 [b]	125 ± 1 [b]	122 ± 2 [b]	125 ± 2 [b]	130 ± 3 [b]	143 ± 5 [a]	143 ± 4 [a]	126 ± 4 [b]	128 ± 4 [b]	141 ± 3 [a]
Left ventricular diastolic stiffness constant (κ)	22.0 ± 0.8 [b]	21.8 ± 0.6 [b]	23.3 ± 0.9 [b]	22.8 ± 0.8 [b]	21.9 ± 0.5 [b]	28.9 ± 0.8 [a]	28.5 ± 0.7 [a]	23.4 ± 1.1 [b]	23.5 ± 0.7 [b]	27.9 ± 0.9 [a]
Left ventricle collagen area, %	11 ± 1 [c]	13 ± 1 [c]	12 ± 1 [c]	15 ± 1 [c]	16 ± 1 [c]	38 ± 2 [a]	22 ± 2 [b]	24 ± 2 [b]	22 ± 2 [b]	27 ± 1 [b]

Values are presented as mean ± SEM, *n* = 10–12 (*n* = 4 for plasma curcumin). Means in a row with unlike superscripts (a, b, c, d, e or f) differ, *p* < 0.05. ALT, alanine transaminase; AST, aspartate transaminase; C, corn starch diet-fed rats; CC5, corn starch diet-fed rats given 5 mg/kg/day curcumin; CC100, corn starch diet-fed rats given 100 mg/kg/day curcumin; CCNP, corn starch diet-fed rats given 5 mg/kg/day curcumin nanoparticles; CBNP, corn starch diet-fed rats given blank nanoparticles; H, high-carbohydrate, high-fat diet-fed rats; HC5, high-carbohydrate, high-fat diet-fed rats given 5 mg/kg/day curcumin; HC100, high-carbohydrate, high-fat diet-fed rats given 100 mg/kg/day curcumin; HCNP, high-carbohydrate, high-fat diet-fed rats given 5 mg/kg/day curcumin nanoparticles; HBNP, high-carbohydrate, high-fat diet-fed rats given blank nanoparticles; RER, respiratory exchange ratio.

3.2. Metabolic Changes

There were no differences between C and H rats in heat produced and RER (Table 1). CCNP rats had lower heat production than C rats, while HCNP and HBNP rats had lower heat production than H rats (Table 1). Total abdominal fat was higher in HBNP rats compared to all other groups. H, HC5, HC100 and HCNP rats had similar fat weight, which was higher than all C diet-fed groups (Table 1). Plasma triglyceride concentrations were higher in H diet-fed groups compared to C diet-fed groups. Plasma total cholesterol concentrations were highest in HBNP group and lower in CC5, CC100, H and HC100 rats, while other groups were intermediate (Table 1). Plasma non-esterified fatty acids were higher in H diet-fed rats compared to C diet-fed rats. C and H rats had similar basal blood glucose concentrations. None of the interventions reduced basal blood glucose concentrations (Table 1). The blood glucose area under the curve was not different between H diet-fed rats (Table 1).

3.3. Cardiovascular Changes

After eight weeks, systolic blood pressure of H diet-fed groups (H, HC5, HC100, HCNP and HBNP) was higher than C diet-fed groups (C, CC5, CC100, CCNP and CBNP) (Table 1). After 16 weeks, systolic blood pressure in H rats was higher than in C rats. None of the interventions affected systolic blood pressure in C diet-fed groups. HC100 and HCNP had normalised systolic blood pressure, whereas HC5 and HBNP had no change in systolic blood pressure compared to H rats (Table 1). Diastolic stiffness was higher in H rats compared to C rats. HC100 and HCNP showed normalised diastolic stiffness, whereas other intervention groups did not change compared to their respective controls (Table 1). Lef ventricular weights with septum were unchanged among all groups whereas right ventricular wet weights were lower in CC5, CCNP and HCNP groups (Table 1). Left ventricles from H rats showed infiltration of inflammatory cells (Figure 1F) and collagen deposition (Figure 2F) whereas these changes were not seen in left ventricles from C rats (Figure 1A and 2A; Table 1). Left ventricles from HCNP (Figure 1I) and HBNP (Figure 1J) rats showed decreased infiltration of inflammatory cells, whereas HC5 (Figure 2G), HC100 (Figure 2H) and HCNP (Figure 2I) showed decreased collagen deposition compared to H rats (Figures 1F and 2F).
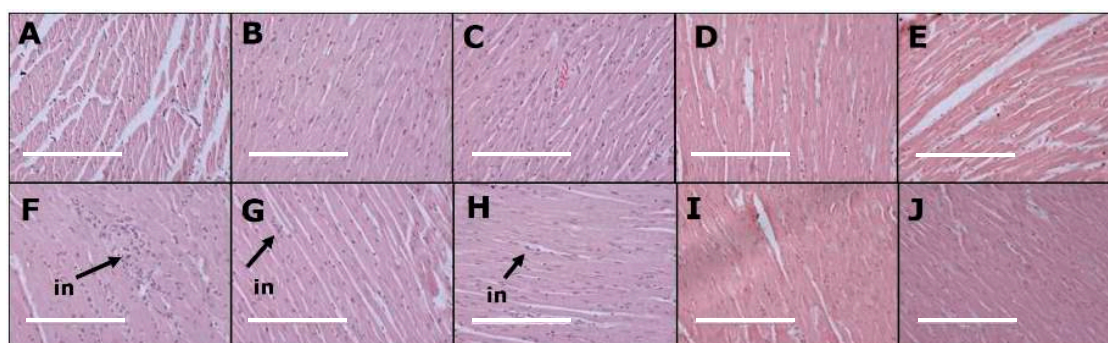

Figure 1. Effects of curcumin on inflammatory cells ("in") in the heart using haematoxylin and eosin stain in corn starch diet-fed rats (**A**), corn starch diet-fed rats given 5 mg/kg/day curcumin (**B**), corn starch diet-fed rats given 100 mg/kg/day curcumin (**C**), corn starch diet-fed rats given 5 mg/kg/day curcumin nanoparticles (**D**), corn starch diet-fed rats given blank nanoparticles (**E**), high-carbohydrate, high-fat diet-fed rats (**F**), high-carbohydrate, high-fat diet-fed rats given 5 mg/kg/day curcumin (**G**), high-carbohydrate, high-fat diet-fed rats given 100 mg/kg/day curcumin (**H**), high-carbohydrate, high-fat diet-fed rats given 5 mg/kg/day curcumin nanoparticles (**I**) and high-carbohydrate, high-fat diet-fed rats given blank nanoparticles (**J**). The white bar is 200 μm.
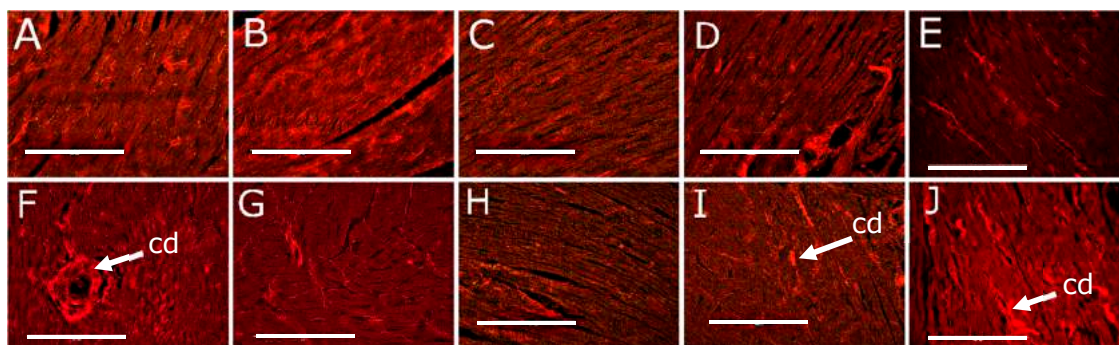
Figure 2. Effects of curcumin on collagen deposition ("cd") in the heart using picrosirius red stain in corn starch diet-fed rats (**A**), corn starch diet-fed rats given 5 mg/kg/day curcumin (**B**), corn starch diet-fed rats given 100 mg/kg/day curcumin (**C**), corn starch diet-fed rats given 5 mg/kg/day curcumin nanoparticles (**D**), corn starch diet-fed rats given blank nanoparticles (**E**), high-carbohydrate, high-fat diet-fed rats (**F**), high-carbohydrate, high-fat diet-fed rats given 5 mg/kg/day curcumin (**G**), high-carbohydrate, high-fat diet-fed rats given 100 mg/kg/day curcumin (**H**), high-carbohydrate, high-fat diet-fed rats given 5 mg/kg/day curcumin nanoparticles (**I**) and high-carbohydrate, high-fat diet-fed rats given blank nanoparticles (**J**). The white bar is 200 μm.

3.4. Liver Changes

Livers from H rats (Figure 3F) showed increased fat deposition and infiltration of inflammatory cells compared to livers from C rats (Figure 3A). HC100 (Figure 3H) and HCNP (Figure 3I) rats had reduced fat deposition compared to H (Figure 3F), HC5 (Figure 3G) and HBNP rats (Figure 3J). Plasma activities of ALT and AST were not different between the groups (Table 1).
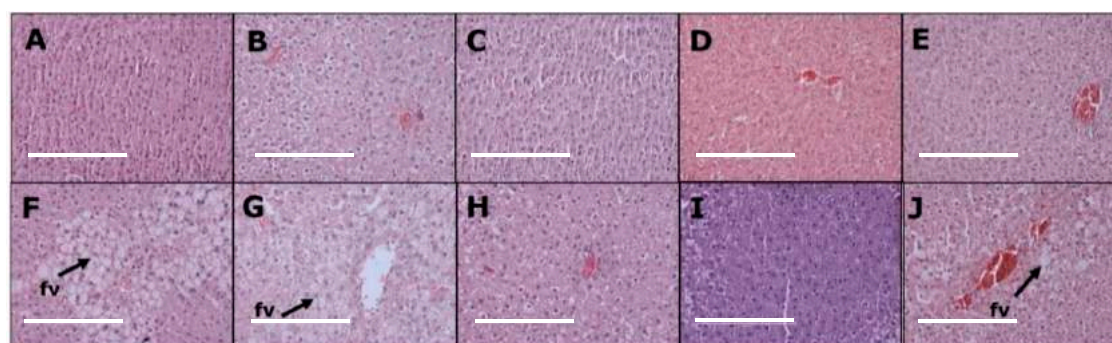
Figure 3. Effects of curcumin on fat deposition ("fv") in the liver using haematoxylin and eosin stain in corn starch diet-fed rats (**A**), corn starch diet-fed rats given 5 mg/kg/day curcumin (**B**), corn starch diet-fed rats given 100 mg/kg/day curcumin (**C**), corn starch diet-fed rats given 5 mg/kg/day curcumin nanoparticles (**D**), corn starch diet-fed rats given blank nanoparticles (**E**), high-carbohydrate, high-fat diet-fed rats (**F**), high-carbohydrate, high-fat diet-fed rats given 5 mg/kg/day curcumin (**G**), high-carbohydrate, high-fat diet-fed rats given 100 mg/kg/day curcumin (**H**), high-carbohydrate, high-fat diet-fed rats given 5 mg/kg/day curcumin nanoparticles (**I**) and high-carbohydrate, high-fat diet-fed rats given blank nanoparticles (**J**). The white bar is 200 *upμ*m.

3.5. Gut Structure and Microbiome

Histology of ileum and colon did not show any abnormalities in the experimental groups demonstrated by normal crypt depth, villi length, goblet cells and lack of inflammatory cell infiltration (Figures 4 and 5).
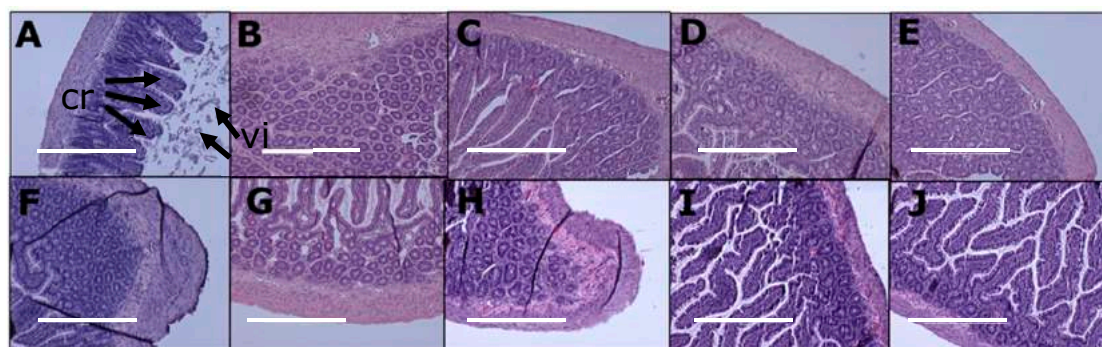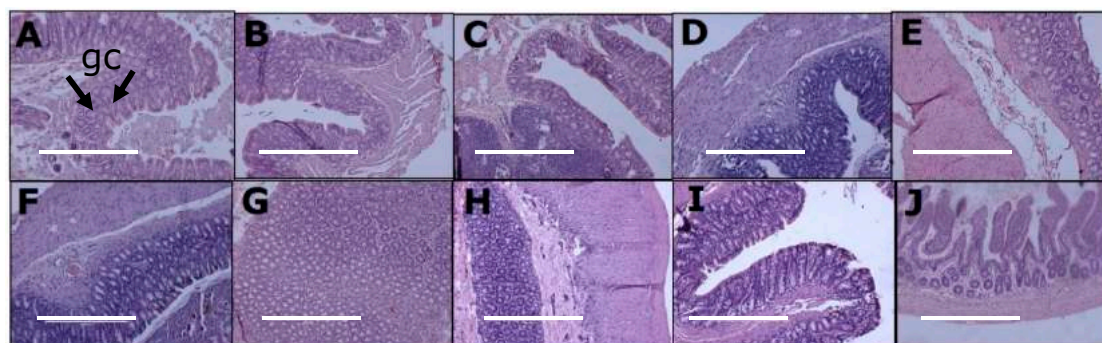
Figure 4. Effects of curcumin on structure, crypt depth ("cr") and villi length ("vi") in the ileum using haematoxylin and eosin stain in corn starch diet-fed rats (**A**), corn starch diet-fed rats given 5 mg/kg/day curcumin (**B**), corn starch diet-fed rats given 100 mg/kg/day curcumin (**C**), corn starch diet-fed rats given 5 mg/kg/day curcumin nanoparticles (**D**), corn starch diet-fed rats given blank nanoparticles (**E**), high-carbohydrate, high-fat diet-fed rats (**F**), high-carbohydrate, high-fat diet-fed rats given 5 mg/kg/day curcumin (**G**), high-carbohydrate, high-fat diet-fed rats given 100 mg/kg/day curcumin (**H**), high-carbohydrate, high-fat diet-fed rats given 5 mg/kg/day curcumin nanoparticles (**I**) and high-carbohydrate, high-fat diet-fed rats given blank nanoparticles (**J**). The white bar is 200 μm.

Figure 5. Effects of curcumin on structure and goblet cells ("gc") in the colon using haematoxylin and eosin stain in corn starch diet-fed rats (**A**), corn starch diet-fed rats given 5 mg/kg/day curcumin (**B**), corn starch diet-fed rats given 100 mg/kg/day curcumin (**C**), corn starch diet-fed rats given 5 mg/kg/day curcumin nanoparticles (**D**), corn starch diet-fed rats given blank nanoparticles (E), high-carbohydrate, high-fat diet-fed rats (**F**), high-carbohydrate, high-fat diet-fed rats given 5 mg/kg/day curcumin (**G**), high-carbohydrate, high-fat diet-fed rats given 100 mg/kg/day curcumin (**H**), high-carbohydrate, high-fat diet-fed rats given 5 mg/kg/day curcumin nanoparticles (**I**) and high-carbohydrate, high-fat diet-fed rats given blank nanoparticles (**J**). The white bar is 200 μm.

In contrast to the histological findings, the high-carbohydrate, high-fat diet changed the colonic bacteria with increases in relative abundance of *Firmicutes* and decreases in relative abundance of *Bacteroidetes* in H rats compared to C rats (Figure 6). Moreover, H rats showed disappearance of *Actinobacteria* compared to C rats (Figure 6). Intervention with 100 mg/kg/day curcumin or curcumin nanoparticles did not change relative abundance of microbiota when compared to H rats (Figure 7).
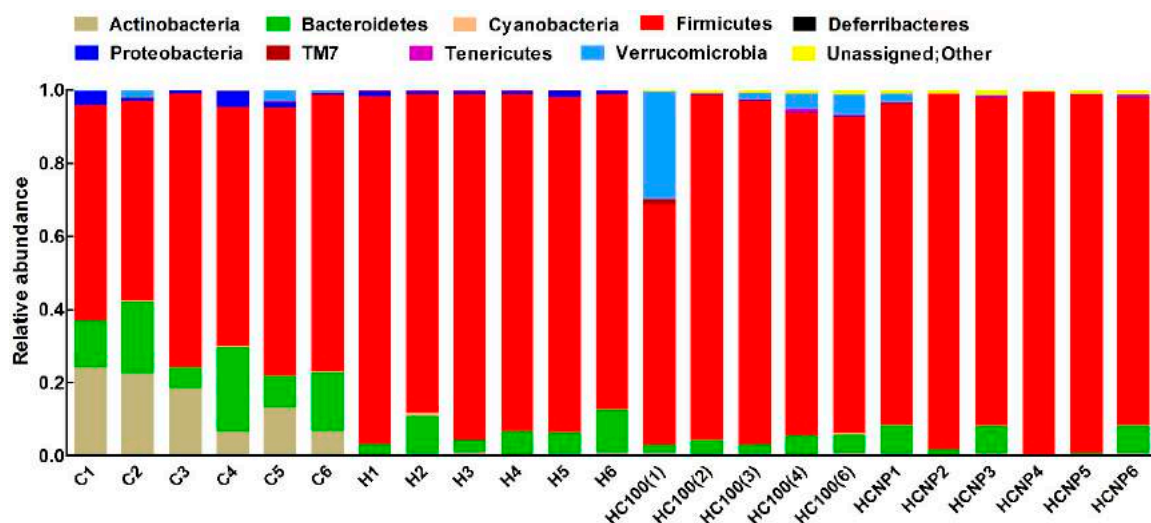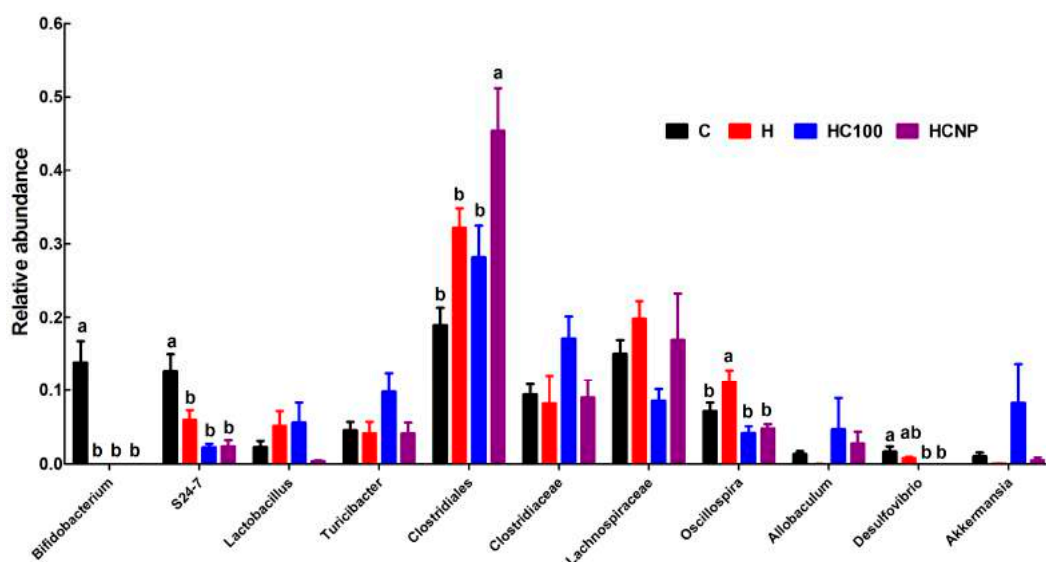
Figure 6. Effects of curcumin on gut microbiota at phylum level. C, corn starch diet-fed rats; H, high-carbohydrate, high-fat diet-fed rats; HC100, high-carbohydrate, high-fat diet-fed rats given 100 mg/kg/day curcumin; HCNP, high-carbohydrate, high-fat diet-fed rats given 5 mg/kg/day curcumin nanoparticles.

Figure 7. Effects of curcumin on gut microbiota at genus level. C, corn starch diet-fed rats; H, high-carbohydrate, high-fat diet-fed rats; HC100, high-carbohydrate, high-fat diet-fed rats given 100 mg/kg/day curcumin; HCNP, high-carbohydrate, high-fat diet-fed rats given 5 mg/kg/day curcumin nanoparticles.

4. Discussion

Both inflammation and oxidative stress are underlying causes of metabolic syndrome leading to an increased risk of developing cardiovascular disease and type 2 diabetes [14]. It has therefore been suggested that compounds such as curcumin may attenuate this syndrome [1], since preclinical studies [26] and clinical trials [27,28] have shown that curcumin has potent anti-inflammatory and anti-oxidative properties, although this is disputed [4]. This study shows that curcumin improved cardiovascular structure and function, especially with the normalisation of systolic blood pressure, left ventricular stiffness and left ventricular collagen deposition, in rats with diet-induced metabolic syndrome. Further, liver fat deposition was reduced. These cardiovascular and liver changes were comparable with high-dose curcumin suspension and low-dose curcumin nanoparticles.

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Absorption and bioavailability of hydrophobic compounds such as curcumin can be improved by inclusion in high molecular weight (40–70 kDa) PLGA compared to low molecular weight (5–15 kDa) PLGA [29]. This current study used PLGA (35–40 kDa) to improve the absorption and bioavailability of curcumin. Products containing PLGA are approved by the Food and Drug Administration for human use as PLGA has superior biocompatibility and biodegradable properties [30]. PLGA copolymers are degraded in the body by hydrolytic cleavage of the ester linkages to lactic and glycolic acids. These compounds are easily metabolised in the body by the Krebs cycle and eliminated as carbon dioxide and water [30]. The PLGA nanoparticles used in the current study had a nine-fold improvement in oral bioavailability; they delivered a safe and potentially translatable dose of curcumin, which improved β-cell function [17]. In this study, curcumin PLGA nanoparticles at 5 mg/kg/day produced similar improvements in systolic blood pressure, left ventricular function and liver fat deposition as curcumin aqueous suspension at 100 mg/kg/day; however, both treatments caused minimal changes in metabolic parameters in high-carbohydrate, high-fat diet-fed rats. These physiological responses were accompanied by increased plasma concentrations of curcumin using PLGA nanoparticles even with a 20-fold lower dose.

Reduction in blood pressure by higher doses of curcumin and nanoparticles containing lower doses of curcumin could potentially be the reason for the reduced cardiac insult and hence improved ventricular inflammation and fibrosis. Possible mechanisms for these changes include modulation of vascular tone [31] and altering the predisposition to vascular disease by targeting Uncoupling Protein (UCP) 2 [32]. In middle-aged and older patients, curcumin improved resistance artery endothelial function by increasing nitric oxide bioavailability and reducing oxidative stress [33].

A recent systematic review found that curcumin improved anthropometric measurements in clinical trials in human adults; however, duration of study and dose/formulation are critical [34]. For example, feeding 1.6 g of curcuminoids daily for three months did not change total body fat and visceral fat, but after six months, both had decreased [35]. Curcumin intervention in mice inhibited high-fat diet-induced inflammation in white adipose tissue and activated UCP1 production in brown adipose tissue [36]. The decrease of inflammation in white adipose tissue and increase of energy expenditure through activation of brown adipose tissue are two key approaches to reduce obesity. Curcumin also prevented non-alcoholic steatohepatitis in rats [37]. In this study, we observed reduction in the fat deposition in liver, which is induced by high-carbohydrate, high-fat diet feeding [23]. In mice, a highly dispersible, low-dose curcumin formulation induced brown-like adipocyte formation more effectively than native curcumin [38]. This finding suggests curcumin formulations have effects *in vivo* at low doses, especially through increased energy expenditure. Curcumin reduced obesity and improved glucose sensitivity in rodents but these results have not been validated in human clinical trials [39]. Furthermore, the conflicting results require future trials that are randomised, double-blind and placebo-controlled to enable comparisons between studies [40]. A recent study concluded that the poor quality of the primary trials means that well-designed and long-term trials, using specific curcumin formulations, are required to make definitive conclusions regarding the efficacy of curcumin [41].

Curcumin (20 μmol/L) induced browning of 3T3-L1 and primary adipocytes through inhibition of lipogenesis [42]. Further, intervention with curcumin (500 mg/kg diet for 12 weeks) reduced body weight in high-fat diet-fed mice while not affecting food intake [43]. These results contrast to the current study where there was no change in body weight or abdominal fat mass at lower doses of 5 or 100 mg/kg/day in rats for a shorter period of eight weeks. High-carbohydrate, high-fat diet-fed rats developed impaired glucose tolerance and hyperinsulinaemia [23]. In this study, there were no effects on glucose metabolism during oral glucose tolerance testing. As there were no changes in glucose responses in oral glucose tolerance testing, insulin concentrations and insulin sensitivity were not expected to change.

In a mouse model of colitis-associated colon cancer, curcumin reduced relative abundance of *Clostridiales* and increased the abundance of *Lactobacillales*, *Bifidobacteriales*, *Erisipelotrichales*, *Coriobacteriales* and the putative order YS2 from the Cyanobacteria phylum [44]. Other studies

have identified modulation of gut microbiome by curcumin [45,46]. Gut microbiome has been reported to be a contributing factor in the development of obesity and obesity-related comorbidities [47]. Modulation of gut microbiome by functional foods such as prebiotics, probiotics and polyphenols has contributed to health benefits [48]. It has also been hypothesised that gastrointestinal effects of curcumin may be able to attenuate intestinal and extra-intestinal diseases [49]. Curcumin reduced local inflammation in the gut by altering intestinal barrier function and modulating the release of LPS into circulation suggesting that curcumin, despite its low oral bioavailability, may be able to mediate its effects through local actions in the gut [50]. Similar results were reported in another study where Western diet-induced changes in intestinal barrier function leading to increased endotoxaemia, macrophage activation and subsequent development of glucose intolerance and atherosclerosis were attenuated by curcumin [51]. In this study, curcumin and curcumin nanoparticles were unable to reverse any changes in gut microbiome induced by high-carbohydrate, high-fat diet, possibly because of the lower doses in this study. *Clostridiales* was increased by the curcumin nanoparticles, whereas *Oscillospira* was decreased by curcumin and curcumin nanoparticles.

This preliminary study with curcumin nanoparticles provided useful information in terms of improvement of blood pressure, ventricular hypertrophy, inflammation and fibrosis. The dose of curcumin through nanoparticles is 5 mg/kg/day for rats, which translates to 50 and 90 mg/day for an adult human using the body surface area and scaling equations, respectively [52,53]. The positive responses of low dose curcumin intervention through nanoparticles provides an avenue to test higher doses to potentially identify the potential metabolic effects of curcumin in diet-induced obesity. These future studies with higher doses of curcumin nanoparticles may warrant clinical evaluation in randomised clinical trials to identify the potential of this naturally occurring molecule with low bioavailability. Future studies may also include evaluation of markers such as specific types of inflammatory cells in the tissues causing damage in the heart and liver through immunohistochemistry to understand the selective roles of curcumin in inhibiting inflammation. Further, future studies may also include tissue evaluation of tissue markers of metabolic improvements such as the measurement of glycogen in the liver and muscles and activities of digestive and metabolic enzymes in the gut and liver.

5. Conclusions

PLGA-curcumin nanoparticles increased plasma curcumin concentrations and effectively improved cardiovascular responses and reduced liver fat deposition to similar extents at 20-fold lower doses of curcumin (5 mg/kg/day compared to 100 mg/kg/day), implying an improved oral bioavailability. The results indicate that a dose-response study with curcumin nanoparticles would help in determining the potential benefits of curcumin in metabolic diseases as usual doses would achieve much higher plasma concentrations than a capsule or an aqueous suspension.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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CHAPTER 8. DISCUSSION AND CONCLUSIONS

Seaweed as a commodity

Seaweeds are grown for nutrition and commercial products but are mostly grown and eaten in east and south-east Asia. Typically, people in Japan consume 4-6 g/day of seaweeds which has been linked to a lower incidence and prevalence of obesity and metabolic syndrome (Teas, Baldeon et al. 2009). If foods containing seaweeds can be developed as tasty foods, seaweed consumption might increase in non-traditional countries (Warwicker and Taylor 2012). For example, a nutritionally balanced pizza with small amounts of seaweed (Seagreens® *Ascophyllum nodosum*) which meets nutritional guidelines has been developed with 77-81% of adults and children finding the pizza as good or better than their usual Margherita pizza (Combet, Jarlot et al. 2014). If seaweeds can be incorporated as a common component in the Australian diet, the health benefits may be evident in all age groups from children to the elderly.

Individuals face a range of challenges when attempting to adopt eating more healthier foods including the comfort of their current habit of an unhealthy diet and their friends and family; expected consumption of unhealthy foods in certain situations; relative low cost of unhealthy foods; lack of time to plan, shop, prepare and cook healthy foods; lack of facilities to prepare, cook and store healthy foods; widespread presence of unhealthy foods; lack of knowledge and skills to plan, shop, prepare and cook healthy foods; and lack of motivation to eat healthily (Munt, Partridge et al. 2017). Other barriers to an increased seaweed consumption include lack of availability and affordability. Hence, overcoming these perceptions will rely upon developing affordable seaweed foods that represent value for money in order to move beyond the present niche markets into wider distribution in mainstream food outlets (Birch, Skallerud et al. 2018). Developing convenient and sophisticated seaweed products to appeal to higher educated consumers will lead to increased seaweed consumption (Birch, Skallerud et al. 2019). If individuals adopt healthier diets, we may see a decreased prevalence of obesity in Australia to a similar prevalence as in Japan.

Australia has the advantage of a large coastline of 25,760 km (Nag 2018) with unpolluted waters which are ideal conditions for developing a seaweed industry using related species based on current uses in Asia. Further, Australia is geographically close

to the existing major seaweed markets in Asia. Currently, about 80% of world macroalgal production is used for human consumption, mainly in Asian countries such as China, Japan and Korea (Capuzzo and McKie 2016). The remaining 20% is used for extraction of phycocolloids, animal feed, fertilisers, water remediation and probiotics in aquaculture (Capuzzo and McKie 2016). If Australia can use its natural advantages for seaweed production, then it may provide seaweed for many uses (Winberg, Ghosh et al. 2009). Importantly, the seaweeds and microalgae studied in this thesis can all be grown in Australia. As at 2014, the Australian seaweed industry was small, based on the harvest of stormcast kelp for alginate and fertiliser, and of introduced species of *Undaria* for the extraction of bioactive compounds (Feng, Wei et al. 2017). The aquaculture of seaweeds specifically for functional foods is likely to be an expensive task. The cost of producing seaweed for health benefits could be reduced by integrating with current commercial uses of seaweeds including biodiesel, biogas, biological charcoal, fertilisers and waste water remediation (Show, Tang et al. 2017) as supporting revenue streams. The demand for seaweed in western markets is expected to increase rapidly due to consumer desire for new protein sources and healthy food supplements in addition to the food industry's interest in sustainable texture additives and food (Kim, Yarish et al. 2017). In 2014, 54,000 tons of seaweed were cultivated in the Americas and Europe with an annual value of 51 million USD, which is less than the value of seaweed products that Korea exported to the United States during the same period (67 million USD) (Kim, Yarish et al. 2017). However, the rapid increase in demand could result in unforeseen ecological and societal consequences, for example with biosecurity lapses allowing the introduction of disease and non-indigenous pests and pathogens, as well as the increased use of algicides and pesticides (Braun 2016).

Applications of seaweeds

Seaweeds are a source of micronutrients and macronutrients with demonstrated nutritional and therapeutic benefits against obesity, type 2 diabetes and cardiovascular disease (Cherry, O'Hara et al. 2019). In this thesis, the main compounds of interest from seaweeds were polysaccharides and proteins which can be used to attenuate metabolic syndrome. To a lesser extent, the fatty acids and carotenoids could provide additive effects. The whole seaweed was effective and hence, future studies with animal or humans should focus on the whole seaweed. This would be consistent

with seaweed consumption in south-east Asian countries as the whole seaweed is eaten (Sharifuddin, Chin et al. 2015). Their acceptance of seaweed as a food source is partly due to the health benefits. During 1993, 97,000 tonnes (dry weight) of seaweed were eaten in Japan, in contrast to only 70 tonnes in Europe (Darcy-Vrillon 1993). Although this is reasonably old data as current comparative data were not available, Japanese seaweed consumption had remained relatively consistent from 1955 (4.3 g/day) to 1995 (5.3 g/day). Seaweed nutritional composition varies according to the species and family. Some red seaweeds contain high protein (47% for *Porphyra yezoensis* and 35% for *Palmaria palmata* as dry weight) and high proportions of polysaccharides (30-71% of dry weight). Furthermore, some macroalgae contain high concentrations of minerals of nutritional value such as calcium and magnesium. For example, the green algae (*Ulva* species) contains up to 3.25 g of calcium/kg of dry weight (MacArtain, Gill et al. 2007). The brown seaweeds, especially the species, *Laminaria digitata*, are rich in iodine with up to 9 g/kg of dry weight (Schiener, Black et al. 2015). Therefore, given the favourable nutritional composition as rich in protein, fibres and mineral content of seaweed, it is difficult to understand the lack of development and progression in the use of seaweeds as food products in Western countries (Fleurence, Morançais et al. 2012). Seaweed was important in ancient cultures such as Ireland and Scotland but the loss of interest could be because eating seaweed was seen as a sign of extreme poverty in the 18th and 19th centuries (Magan 2019, BioMara n.d). It may also be due to the lack of knowledge in the preparation of seaweeds in countries and regions such as USA, Europe and Australia. A direct source would be advantageous even though an indirect source of seaweed consumption in humans is by using algae as animal feed as with microalgae fed to fish to increase omega-3 fatty acid concentrations (Tocher, Betancor et al. 2019).

Commercial uses of seaweeds to offset the cost of producing functional foods include seaweeds grown in industrial waste water for bioenergy and bioremediation applications. For example, *Ulva* species have been used for bioremediation due to their fast growth rates and broad geographical distribution (Lawton, Mata et al. 2013). Seaweeds have been studied for their biosorption of heavy metals from coal-fired power stations, for example, their ability to grow in and concentrate a group of heavy metals from industrial waters (Saunders, Paul et al. 2012). Another example is the *Oedogonium* species which have been grown in metal-contaminated waste water to generate biomass for bioenergy applications and concomitantly bioremediate water

(Roberts, de Nys et al. 2013). Furthermore, this species can be stressed to increase growth, with the addition of CO_2 to alter pH level shown to increase growth of algal biomass by approximately three-fold from 6.8 g dry weight/m^2/day to 22.5 g dry weight/m^2/day (Roberts, de Nys et al. 2013).

The red macroalga, *Asparagopsis taxiformis*, reduced methane production during *in vitro* fermentation with rumen fluid (Machado, Magnusson et al. 2014). This species grows prolifically off the Queensland coast in Australia. Cows are known to eat seaweed and feeding <2% of dry *A. taxiformis* reduced the microbes in the cows' stomachs that cause them to burp when they eat grass, releasing methane. It also decreased fermentation and volatile fatty acid production. If Australia could grow enough seaweed for every cow in Australia, it could reduce the country's greenhouse gas emissions by 10% (2019). Methane is a far more active greenhouse gas than carbon dioxide and therefore *A. taxiformis* may be useful in helping to slow climate change.

Advantages of seaweeds compared to traditional crops

Producing seaweeds for their nutritional and bioactive properties has several advantages over traditional crops such as cereals. Seaweeds require less land, have higher yields, do not degrade soils, provide additional sources of income for regional communities, and grow in droughts especially if municipal or industrial waste-water has been used (Nabti, Jha et al. 2017). For example, maximal seaweed production is estimated as 14.31 g of dry weight/m^2/day at a photosynthetic efficiency of 8% (Grobbelaar 2010) which would translate to 52,231 kg/hectare/year. In contrast, the average cereal yield in the USA was approximately 6-fold lower at 8,281 kg/hectare in 2017 (2017).

Seaweeds are of high nutritional value including as a source of fibre and protein. People in Western countries consume foods with low fibre content, so that mean dietary fibre intake is decreased; for example, fibre intake in the USA during 1999-2008 was 13.1-16.1 g/day (King, Mainous et al. 2012). This contrasts with the American Dietetic Association recommendation for a daily dietary fibre intake of 25 g for adult females and 38 g for adult males (Slavin 2008). The well-established health benefits of fibre include lower serum cholesterol concentrations, lower coronary heart disease risk, reduced blood pressure, enhanced weight control, better glycaemic control, reduced risk of some cancers and improved gastrointestinal function (Anderson, Smith et al. 1994). Current guidelines to meet this recommended fibre

intake are increased servings of fruit, vegetables, whole grains, dried beans and peas (Anderson, Smith et al. 1994). Seaweeds could provide a major proportion of the recommended fibre intake. From this thesis, *Sargassum siliquosum* contained 41.4% fibre, *Sarconema filiforme* contained 21.7% fibre and *Caulerpa lentillifera* contained 17.5% fibre. Therefore, a 30 g serving of *S. siliquosum* will provide half of the recommended fibre intake for females and a third for males. This supports the role of functional foods in providing a benefit in addition to a healthy diet.

Functional foods and nutraceuticals

Functional foods are foods or beverages that have a physiological benefit that enhances overall health, and helps or prevents a disease condition (Hasler 2002). They are similar in appearance to conventional foods and consumed as part of a usual diet (Hasler 2002). Nutraceuticals as products isolated or purified from foods are generally sold in medicinal forms such as tablets not usually defined as a food (Aronson 2017). A nutraceutical has demonstrated physiological benefits or provides protection against chronic disease (Aronson 2017).

If the whole food can provide a benefit at a realistic dose, then a functional food should be preferred over a nutraceutical. This follows the concept of food having more meaning than just to meet nutrition and energy requirements. For example, it is important that the use of food is not simply replaced by a tablet (i.e., a "polypill") which treats symptoms or diseases by decreasing blood pressure, reducing body weight or improving dyslipidaemia. Furthermore, if functional foods can be incorporated as part of a balanced diet and exercise routine, there is a higher likelihood of increased compliance than with people eating whatever they want and taking a tablet and thinking it will reverse lifestyle decisions to improve their health as a "magic bullet" concept. Keeping the responsibility on the individual should not be compromised. This contrasts to the medical model of disease where medication is administrated to treat specific symptoms, often not treating the whole disease state, for example in metabolic syndrome. Locally-grown seaweeds can be developed into low-cost sustainable functional foods which address fibre and protein deficiencies.

Microalgae can also provide benefits owing to their fast growth rate and ability to accumulate high levels of bioactives such as eicosapentaenoic acid and protein. Microalga growth can be further increased by altering their environment including pH, salinity, CO_2 concentration and light. Innovative food production strategies are

required to meet the world population growth to a predicted total of 9.8 billion people by 2050. Furthermore, providing food rich in macronutrients is also important. For example, in 2007, it was reported that 1 billion people (~15% of the then current population) were protein-deficient (Wijnands, van der Meulen et al. 2007). As plant-based proteins account for the majority of protein intake worldwide, it is important to cultivate plants with a high protein content. The mechanisms for the reported effects are related to the effect on the fibre and the prebiotic effect which modulates the gut microbiome. In the last decade, there has been an explosion in the understanding of the gut microbiome (Amon and Sanderson 2017).

Seaweeds and freshwater algae are a source of fibre and protein. This thesis has provided evidence for therapeutic uses demonstrated by seaweeds improving metabolic syndrome. Polyphenols and natural products have demonstrated the ability to improve the health of rats in other chronic inflammatory diseases including inflammatory bowel disease (Ghattamaneni, Panchal et al. 2019), chronic kidney disease (Diwan, Brown et al. 2017) and osteoarthritis (Sekar, Shafie et al. 2017), all of which decrease quality of life in people, especially in the ageing population. If these results on rats can be translated to humans, this would be a good way to improve health.

Metabolic syndrome

A large study involving 195 countries for 25 years found that, although there has been a decrease in death rates since 1990, this did not match up to an expected similar decrease in years lost by disability (Vos, Abajobir et al. 2017). Hence, people are living longer but disability-free years have not increased. Diseases such as diabetes and obesity are not rapidly killing people like aggressive forms of cancer but rather cause a decreased quality of life and economic loss due to unproductive workers. Metabolic syndrome is increasing in prevalence worldwide with current treatment options proving inadequate. There are socioeconomic effects such as increasing personal wealth and obesity, demonstrated by increased obesity rates in the USA and Europe during the mid to late 20th century. This relies on the abundance of cheap and readily available food along with changing social norms (Levine 2011, Hruby and Hu 2015). Diet is well-established as a major contributing factor to obesity and metabolic syndrome (Han and Lean 2016), although, as an apparent paradox, decreased personal wealth is also linked to obesity. Education levels also have a role as college-educated

people have a lower prevalence of obesity (Ogden, Fakhouri et al. 2017). During 2011-2014, the age-adjusted prevalence of obesity among adults was lower in the highest income group (31.2%) than the other groups (40.8% and 39.0%). The age-adjusted prevalence of obesity among college graduates was lower (27.8%) than among those with some college (40.6%) and those who were high school graduates or less (40.0%) (Ogden, Fakhouri et al. 2017). Furthermore, there is also a genetic component, with obesity predisposing genes interacting with the environment to influence development (Choquet and Meyre 2011).

Consumption of a diet high in simple sugars, saturated and *trans* fats is an important factor for the increasing prevalence of metabolic syndrome. If diet is improved, an individual will be of healthy weight with a decreased risk of developing type 2 diabetes and cardiovascular disease, thereby reducing risk years of disability and premature death. Therefore, a high-carbohydrate, high-fat diet-induced metabolic syndrome rat model was used to test the effectiveness of seaweeds as food interventions in preventing or reversing metabolic syndrome. Obesity is a prolonged positive energy imbalance but it is a complex, multifactorial and largely preventable disease (Hruby and Hu 2015). Soluble dietary fibre improved energy homeostasis disrupted by a high-fat diet (Wang, Hong et al. 2018) by modulating the composition of the gut microbiota (Holscher 2017). Macroalgae (*Kappaphycus alvarezii*, *S. filiforme*, *C. lentillifera* and *S. siliquosum*), microalgae (*Nannochloropsis oceanica*) and a curcumin nanoparticle formulation showed the ability of natural products to attenuate symptoms of metabolic syndrome in this thesis. A healthy diet is one that is low-carbohydrate and low-fat, together with fruits, vegetables and protein.

The macroalgae were sourced from all three main phyla; *K. alvarezii* and *S. filiforme* from Rhodophyta (red algae), *C. lentillifera* from Chlorophyta (green algae) and *S. siliquosum* from Phaeophyta (brown algae) to determine if polysaccharides act as fibre to produce therapeutic effects. Current evidence suggests that seaweed polyphenols are a good option for the amelioration of cardiovascular-associated disorders such as metabolic syndrome (Gómez-Guzmán, Rodríguez-Nogales et al. 2018). These macroalgae contained soluble and insoluble fibre as a large proportion of their biomass, ranging from 16.6% to 41.4%, and hence prebiotic effects were observed as reported in the literature (Cherry, Yadav et al. 2019). Red algae contain carrageenans, green algae contain ulvans and brown algae contain fucoidans and alginates. Their chemical structure of the polysaccharides (position of OH and S

groups) does not seem critical to producing prebiotic actions. Most of the interventions produced one or more responses relevant to metabolic syndrome including reduced body weight, decreased inflammation, normalised systolic blood pressure, improved structure and function of heart and liver, and decreased triglycerides and total cholesterol; although none of the interventions improved glucose and insulin intolerance. The likely mechanisms for the improved physiology of the rats were the actions of the polysaccharides as prebiotics. Furthermore, the microbiome of rats changed during the feeding of the obesogenic diet and seaweed interventions modulated the bacterial species. There is strong evidence for the role of the microbiome in health and disease (Sekirov, Russell et al. 2010).

In this thesis, *N. oceanica* increased lean mass in rats healthy corn starch fed controls rats. The intactness of the cell wall of *N. oceanica* likely decreased the bioavailability of both eicosapentaenoic acid and carotenoids which may have produced responses in metabolic syndrome rats. Carotenoids have low bioavailability which will limit their biological responses (Fernández-García, Carvajal-Lérida et al. 2012). Hence, biotechnology can be used to increase bioavailability and biological responses. In the final study of this thesis, curcumin nanoparticles reduced systolic blood pressure to the same extent as a 20-fold higher dose of an unformulated curcumin suspension. The curcumin study showed the application of biotechnology to natural products to increase their therapeutic activity against diseases (Patra, Das et al. 2018). Furthermore, a component of brown seaweed, alginates, has been investigated in the biomedical field to produce nanoparticles because of several favourable characteristics, such as low cost of manufacture, biocompatibility and easy gelling in response to the addition of divalent cations (Patra, Das et al. 2018). Diverting part of a seaweed such as alginate to increase oral bioavailability of another seaweed constituent such as carotenoids may be useful to improve overall responses.

Local seaweeds from regional Australia

Australia is a major agricultural producer and exporter, with this sector accounting for 12% of gross domestic product. The current population of Australia is ~25 million and it is expected to reach 35 million by 2030 (Millar and Roots 2012). The issues of food and nutrition security, food waste and losses are becoming more prominent in society (Parfitt, Barthel et al. 2010). Utilising local resources and limiting

transportation costs are two ways to address some of these issues. One of the highlights of the studies from this thesis was the health benefits of regional produce.

In this thesis, an example is *N. oceanica* sourced from a microalgae farm (http://teraform.com.au/) in Miles, a town in regional Queensland with a population of around 1800 which is 330 km from Brisbane, the capital city of Queensland. This town has the advantages of less expensive non-arable land and a warm climate suited to microalgae. These conditions are ideal for seaweed and microalgal aquaculture. *S. siliquosum* was sourced from Nelly Bay, Magnetic Island, Queensland, a small tourist area with a population of just over 1000 on an island near Queensland's fourth largest city, Townsville (Australian Bureau of Statistics 2016). Macroalgae cover is substantial in this area, between 5-37% and *Sargassum* species cover increased in Nelly Bay from 30% in 2016 to 53% in 2017 (Reef Ecologic 2017). This represents a large quantity which could be used for its health benefits and other applications such as alginates for food industry and biochar for agricultural fertiliser. *K. alvarezii* has been successfully cultivated in Fiji and has been an economic driver for the local community. These algae farms are commonplace in Korea and can be used in Australia to grow a commercially-viable seaweed industry (Walton 2017). The United Nations expects world population to grow to 9.8 billion in 2050 and 11.2 billion in 2100 (Gerland, Raftery et al. 2014). Additionally, this will be an ageing population so it is important to have enough food which is balanced in macro- and micro-nutrients which seaweeds can provide.

Early onset obesity

An interesting area of research is defining how prenatal conditions determine adult life disease risk. The 'Barker hypothesis' proposed that adverse nutrition in early life, including prenatally as measured by birth weight, increased susceptibility of diseases such as metabolic syndrome in adulthood (De Boo and Harding 2006). For example, men who were conceived during the Dutch famine of 1944-1945 had higher rates of obesity at age 19 years than those conceived before or after it (Ravelli, van Der Meulen et al. 1999). The parental environment including diet, body composition, metabolism and stress has been linked to post-natal nutrition and long-time health outcomes (Fleming, Watkins et al. 2018). Therefore, it is important that parental health is optimal to ensure a better probability of offspring health. This links with childhood obesity and evidence where children who are overweight and obese will likely also be

overweight and obese in adulthood (Sahoo, Sahoo et al. 2015). Hence, it is clear that childhood obesity needs to be addressed (Pandita, Sharma et al. 2016).

Currently about one-third of children and adolescents in the United States are classified as overweight or obese (Ogden, Carroll et al. 2014). The prevalence of overweight or obesity increases with advancing age and it is difficult to reverse, often persisting throughout life and increasing risk for other diseases (Lanigan 2018). Therefore, it is important to intervene as early as possible. Children with obesity are at high risk for co-morbidities such as type 2 diabetes, hypertension, dyslipidaemia, obstructive sleep apnoea and non-alcoholic fatty liver disease (Kumar and Kelly 2017). There have been other ideas about the role of metabolic syndrome including the role of circadian rhythm and importance of meal timings (Zimmet, Alberti et al. 2019). It is important to consider multiple aspects of metabolic syndrome given its multifactorial pathogenesis.

Limitations

The focus of this thesis was on a dietary model in rats to mimic human metabolic syndrome. This diet is the most appropriate to develop obesity, diabetes, hypertension and dysfunction of the heart, blood vessels, liver and kidney (Panchal and Brown 2011), but it is a fixed diet, unlike most human diets. The model used 8-9 week old rats which were fed for 16 weeks for a total age of about six months; equivalent to 18 years in humans (Andreollo, Santos et al. 2012). This addresses an important age in humans but does not investigate middle-aged adults who are more likely to be obese or older adults who are more likely to show the chronic diseases related to obesity. Further, all diseases associated with obesity such as skeletomuscular diseases and cancer were not addressed. Seaweeds were fed to the rats as 5% of the total diet. This allowed results to be compared between studies within this thesis. There are large variations in doses within the literature, however using multiple doses of an intervention within each study would provide information to determine the minimal dose where effects occur. Narrative reviews were conducted for the literature review in Chapter 1 and introduction for the animal studies in Chapters 2-6. Systematic reviews and meta-analyses were not performed but these types of reviews are useful for determining specific gaps in the literature and drawing further conclusions from existing studies.

Future directions

Seaweed and microalgae have multiple future applications. As an example, the demand for carrageenan has risen by 5% annually and in 2016 red seaweeds provided 13.3% of the global hydrocolloid market, valued at 762.35 million USD (Diwan, Brown et al. 2017), showing that there is a need to grow more seaweed. Evidence from this thesis showed seaweeds provided numerous benefits by their addition to a high-carbohydrate, high-fat diet-induced metabolic syndrome in rats. Furthermore, seaweed addition to the corn starch group may have a protective effect. Carrageenans have multiple uses, not just as a thickening agent but also as a health food for metabolic syndrome.

Following on from the 'Barker hypothesis' information, the health of the mother before and during pregnancy affects the offspring. Therefore, a rat study could be performed where seaweed is fed to female rats before pregnancy to determine whether the seaweed health benefits are passed onto the offspring, even when fed a high-carbohydrate, high-fat obesogenic diet. This type of generational study is important to provide information to combat increasing childhood obesity. If the current generation of people is healthy, this may be an important factor to predict the health of the next generation.

Randomised, double-blind placebo controlled human clinical trials are the ultimate test of an intervention and are required before gaining approval for general use. Each intervention affected some components of metabolic syndrome. *K. alvarezii* containing κ-carrageenan prevented obesity-associated changes and *S. filiforme* containing ι-carrageenan decreased body weight. These studies showed the effects of carrageenans as a source of fibre which prevents and improves metabolic syndrome symptoms in rats. *C. lentillifera*, currently available on a commercial scale in several countries, had the greatest effect on body weight loss. This is important as losing weight will likely improve other areas of metabolic syndrome. Finally, a curcumin nanoparticle formulation demonstrated the application of biotechnology to overcome low bioavailability. This may have future applications to overcome low carotenoid bioavailability which may increase the anti-oxidant and anti-inflammatory effects. A combination of seaweeds with different compositions will have a synergistic effect and hence be most effective at attenuating metabolic syndrome. *S. siliquosum* also had

fibre and prebiotic effects. The microalgae, *N. oceanica*, was shown to be a good source of protein.

C. lentillifera or sea grapes is probably the most effective seaweed in this study. The parameters for a clinical trial with sea grapes could be a 16-week trial with one group receiving 7.4 g of seaweed per day and another group receiving a placebo. The age group could be 18-35 years as this group have a high prevalence of overweight and obesity. 16-weeks should be long enough to detect physiological effects but not too long that there are compliance issues. The dose of 7.4 g/day is calculated using the Reagan-Shaw equation (Reagan-Shaw, Nihal et al. 2008) for converting rat to human doses (1 g x 7.4= 7.4 g). It would be useful to recruit people who are overweight but not obese (25-30 kg/m^2) and can be mildly hypertensive and not on any medications. There should be 40 people total (considering typical drop-out rates of 10%), the aim would be to recruit 45 participants. This number of participants will allow significant conclusions to be drawn for the study.

An important aspect of biotechnology is drug delivery to deliver drugs to the right tissues, at appropriate times and optimal dosages. In the curcumin nanoparticle study, it was shown that nanoparticle formulation improves bioavailability by 20-fold. This is important and linked to seaweeds as the polysaccharide components have been used in nanoparticle formulation due to their biocompatibility, lower cost and increased bioavailability. Other components of seaweeds such as fucoxanthin pigments have low bioavailability.

Conclusions

Metabolic syndrome diagnosis requires three of five symptoms. Improving all symptoms to normal reference values must be the goal although improvement in one parameter such as weight loss will undoubtably be of benefit. For example, it has been reported that even modest (10% weight loss over one year; 9.5 kg for an average 1.78 m male weighing 95 kg with a BMI of 30 kg/m^2) weight loss achieved with diet and exercise will improve other components of metabolic syndrome such as blood pressure (Wagh and Stone 2004). Hence, the effects on other components in the rat model used in this thesis may take longer than the eight weeks of intervention to be detectable. The advantages of using rat models to study metabolic syndrome is the ability to monitor histological, functional, biochemical and morphological changes which is difficult to accomplish in humans (Wong, Chin et al. 2016). The model should be

reproducible, simple, reliable and affordable with minimal disadvantages (Wong, Chin et al. 2016). This diet-induced model used throughout the current thesis has demonstrated these characteristics with the additional capability of being reversible, in that the organ damage has not progressed to a stage where it is permanent.

CHAPTER 9. REFERENCES

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