POTENTIAL OF ALGAE EXTRACTS FOR THE ATTENUATION OF DIET-INDUCED METABOLIC SYNDROME

A thesis submitted by

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ABSTRACT

Metabolic syndrome is the clustering of multiple symptoms that directly increase the risk of developing cardiovascular disease, type 2 diabetes and non-alcoholic fatty liver disease. It is estimated that one quarter of the world's adult population has metabolic syndrome and rates of obesity have doubled in 70 countries between 1980 and 2015. Inducing weight loss and improving cardiometabolic health using food-based products is an attractive alternative to pharmaceutical treatment, as they are generally cheaper to produce and do not possess serious side effects. Edible algae have been consumed for thousands of years and remain a staple of the Japanese diet in particular to this day. Despite this, algae are rarely used as a health or food resource in Australia even though many species of algae are abundant along Australian coasts. Identifying the health benefits of Queensland-grown algae could lead to the development of high-value products to improve human health.

The purpose of this PhD project was to determine whether extracts from three different algal species grown in north Queensland can attenuate diet-induced metabolic syndrome in rats. The advantages of rats in biomedical research are their anatomical and physiological similarities to humans, short lifespan, and ease of maintenance that allows for the testing of nutraceutical and pharmaceutical treatments in a pre-clinical trial (Hashway & Wilding 2020). This can help to determine whether a costly clinical trial is worth attempting, and guide dose selection and timing. The algal extracts of interest to this thesis included a highly sulphated soluble fibre known as ulvan, and two different carotenoid pigments called astaxanthin and fucoxanthin. To test the efficacy and safety of these algal extracts, a rat model using a validated high-carbohydrate, high-fat diet was used to mimic the human obesogenic diet. This obesogenic diet is characterised by an increased intake of saturated fat and simple carbohydrates and is linked to the development of obesity and the other symptoms of metabolic syndrome including high blood pressure, glucose intolerance, dyslipidaemia and fatty liver.

Young, male Wistar rats were fed with the high-carbohydrate, high-fat diet for 16 weeks and a corn-starch-based diet was used to induce a lean control. The algal extracts were introduced into both of these diets at the 8-week mark using a low and a high dose to

assess whether they could reverse or prevent further development of the symptoms of metabolic syndrome.

Chapter 1 is a literature review of the characteristics of algae that highlight their potential for commercial success whilst simultaneously benefiting the environment. Furthermore, the therapeutic potential of the major algal extracts is explored. Chapter 2 is the description of the materials and methods used for the animal studies and analysis.

Chapter 3 demonstrated that ulvan extracted from *Ulva ohnoi* slowed the progression of obesity by lowering body weight gain by 33% and fat mass gain by 36 - 45%, and through decreasing overall fat deposition in the liver. This anti-obesity effect was correlated with gut microbiome changes and a 25% reduction in feed efficiency. The Firmicutes/Bacteroidetes ratio was reduced from 64 to 11, and in particular *Alistipes* spp. and *Prevotella* spp. were increased in abundance. Chapter 4 investigated fucoxanthin extracted from *Phaeodactylum tricornutum* and demonstrated a normalisation of systolic blood pressure matching the corn-starch control (20 mm Hg reduction) and reduced fat deposition in the liver. While there were beneficial changes to serum lipids, including a 42% reduction to triglycerides and a doubling of high-density lipoprotein cholesterol, there was also a 117% elevation of low-density lipoprotein that increases the risk of cardiovascular disease. Chapter 5 discusses the response of obese rats to astaxanthin. It was found that a comparatively low dose of 1.2 mg/kg body weight/day was not effective in attenuating metabolic syndrome apart from a 34% reduction to serum triglycerides. This dose was selected based on the success of clinical trials testing a dose of 12 mg/day in humans, which is an equivalent dose to the one chosen for this study using the Reagan-Shaw formula for dose conversion.

Overall, the animal studies conducted found that tropical Australian algae extracts, particularly ulvan and fucoxanthin, have potential for the treatment of metabolic syndrome. While astaxanthin has shown success in previous human and animal studies, it is likely that the dose used in this thesis was too low to produce an effect. Further work is needed to test these algal extracts in clinical studies to determine whether these health benefits can be translated to humans. A major challenge in translation is the high variability in algae biomass content and even the structure of algae extracts such as ulvan, in response

to environmental variability and extraction technique. It is important that these factors are considered and reported on when designing future studies.

CERTIFICATION OF THESIS

This thesis is entirely the work of Jessica Pahl except where otherwise acknowledged. The work is original and has not previously been submitted for any other award, except where acknowledged.

Principal Supervisor: Dr Paulomi (Polly) Burey

Associate Supervisor: Professor Lindsay Brown

Associate Supervisor: Dr Sunil Panchal

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Student and supervisor's signatures of endorsement are held at the university.

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ASSOCIATED PUBLICATIONS

Du Preez, R, Pahl, J, Arora, M, Ravi Kumar, M, Brown, L & Panchal, SK 2019, 'Low-dose curcumin nanoparticles normalise blood pressure in male Wistar rats with diet-induced metabolic syndrome', *Nutrients*, vol. 11, no. 7, article no. 1542.

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AUC	Area under the curve
AUD	Australian dollar
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
C	Corn starch diet-fed control rats
CAH	Corn starch diet-fed rats supplemented with 1.2 mg/kg body weight/day fucoxanthin
CAL	Corn starch diet-fed rats supplemented with 0.6 mg/kg body weight/day fucoxanthin
CFH	Corn starch diet-fed rats supplemented with 6.5 mg/kg body weight/day fucoxanthin
CFL	Corn starch diet-fed rats supplemented with 0.65 mg/kg body weight/day fucoxanthin
CS	Corn starch
CUH	Corn starch diet-fed rats supplemented with 2% ulvan
CUL	Corn starch diet-fed rats supplemented with 0.5% ulvan
DEXA	Dual-energy x-ray absorptiometry
EPA	Eicosapentaenoic acid
FAO	Food and Agriculture Organization
F/B	Firmicutes/Bacteroidetes ratio
FTIR	Fourier transform infrared
H	High-carbohydrate, high-fat diet-fed control rats
Ha	Hectare
HAH	High-carbohydrate, high-fat diet-fed rats supplemented with 1.2 mg/kg body weight/day fucoxanthin
HAL	High-carbohydrate, high-fat diet-fed rats supplemented with 0.6 mg/kg body weight/day fucoxanthin
HCHF	High-carbohydrate, high-fat
HDL	High-density lipoprotein
HFH	High-carbohydrate, high-fat diet-fed rats supplemented with 6.5 mg/kg body weight/day fucoxanthin
HFL	High-carbohydrate, high-fat diet-fed rats supplemented with 0.65 mg/kg body weight/day fucoxanthin
HSD	Honestly significant difference
HUH	High-carbohydrate, high-fat diet-fed rats supplemented with 2% ulvan
HUL	High-carbohydrate, high-fat diet-fed rats supplemented with 0.5% ulvan
JCU	James Cook University
LDL	Low-density lipoprotein
LPS	Lipopolysaccharides
MCT	Medium-chain triglycerides
NMDS	Non-metric multidimensional scaling

OGTT	Oral glucose tolerance test
OTUs	Operational taxonomic units
PCR	Polymerase chain reaction
SBP	Systolic blood pressure
SEM	Standard error of the mean
t	tonne
USQ	University of Southern Queensland
VLDL	Very low-density lipoprotein

CHAPTER 1 – INTRODUCTION

1.1 Background on algae

Algae encompass a large, diverse population of photosynthetic organisms found in both freshwater and seawater, which can be further divided into either multicellular (macroalgae) or unicellular (microalgae) subtypes. Macroalgae are generally divided into three taxonomic groups: Chlorophyta (green), Phaeophyta (brown) and Rhodophyta (red). The algae population includes approximately 72,500 different species, of which more than 20,000 are classified as diatoms (unicellular eukaryotic microalgae) (Guiry 2012). Understandably, this vast range of algae contain a large variety of compounds including polysaccharides, proteins, fatty acids and minerals as well as secondary metabolites such as carotenoids and polyphenols (Cardozo et al. 2007).

Seaweeds do not have a formal definition but are usually considered as marine macroalgae as they grow in seawater or brackish water. Seaweeds are fundamental to marine ecological systems as they provide a major contribution to the structure and energy that supports biodiversity (Wernberg et al. 2019). This contribution includes providing three-dimensional structure, biomass, habitat, and food for associated species, particularly in the intertidal and subtidal rocky reefs (Schiel & Foster 2006). They are rapidly growing and are distributed across at least 25% of the world's coastline in the intertidal and subtidal zones where there is sufficient light to support photosynthesis (Wernberg et al. 2019).

Seaweed has been consumed as a staple food in many countries across the world, notably Japan, Korea, China, Ireland and Hawaii (Pérez-Lloréns et al. 2020). Harvesting seaweed in Asian countries has been a common practice for thousands of years due to the easy availability along coastlines. It was a task often completed by women, so was particularly important at times when the male population had to travel from home due to conflict or to find work (Pérez-Lloréns et al. 2020). Harvesting seaweed is also of great importance during times of famine, most notably in Ireland during the Great Famine, and is still seen as marker of poverty today (Pérez-Lloréns et al. 2020). In addition to the traditional use of algae as a foodstuff (Mouritsen, Rhatigan & Pérez-Lloréns 2019), it was also revered

in some countries for its medicinal uses and this is evident in historical medical texts. For example, Liu et al. (2012) reported that it was noted in a Chinese medical book from 1578 that a red macroalgae called *Sargassum* could be used to "soften hard lumps, dispel nodes, eliminate phlegm and induce urination in humans". A longstanding Korean tradition is to consume seaweed soup for several months after giving birth to supply vitamins and minerals such as iodine and iron to help with recovery. Other historical uses include use as a fertiliser, animal fodder and using the gelling properties of some algal species to thicken food products (Pérez-Lloréns et al. 2020). Due to this rich history of algae, it is not surprising that these traditional uses are encouraging new investigation into their uses in current society.

The Food and Agriculture Organization (FAO) of the United Nations reported that world production of macroalgae more than tripled from the year 2000 to 2018, particularly in Asian countries such as China and Indonesia which account for 81% of total global macroalgae production (Table 1) (FAO 2020). Seaweed species used primarily as human food, including Japanese kelp, wakame, nori and laver, have all been steadily increasing in production and China is the biggest producer of these varieties (Table 1) (Ferdouse et al. 2018). Edible seaweeds have a rich composition of micronutrients including iodine, iron, zinc, copper, magnesium, potassium, calcium, and vitamins B12, A and K (Ferdouse et al. 2018). Seaweed is an important component of the Japanese diet and their micronutrient intake, as it is found in approximately one-fifth of Japanese meals (Cherry et al. 2019). The higher consumption of polyunsaturated fatty acids, minerals, vitamins and fibre found in the Japanese diet, partially attributed to the high seaweed intake, is linked to a lower risk for all-cause, cancer and cardiovascular mortalities compared to the typical Westernised dietary pattern (Nanri et al. 2017).

Seaweeds are also important in food ingredient production. In Indonesia in particular, farming of tropical seaweed species such as *Kappaphycus alvarezii* and *Eucheuma* spp. has been increasing due to the rising demand for the polysaccharide, carrageenan (FAO 2020; Ferdouse et al. 2018). Carrageenan and other algal phycocolloids such as agar and alginate are used predominantly as texturising agents in foods and beverages.

Table 1: World aquaculture production of aquatic algae (FAO 2020)

	2000	2005	2010	2015	2016	2017	2018
	(thousand tonnes, live weight)						
Japanese kelp (*Laminaria japonica*)	5 380.9	5 699.1	6 525.6	10 302.7	10 662.6	11 174.5	11 448.3
Eucheuma seaweeds nei[1] (*Eucheuma* spp.)	215.3	986.9	3 479.5	10 189.8	9 775.9	9 578.0	9 237.5
Gracilaria seaweeds (*Gracilaria* spp.)	55.5	933.2	1 657.1	3 767.0	4 248.9	4 174.2	3 454.8
Wakame (*Undaria pinnatifida*)	311.1	2 439.7	1 505.1	2 215.6	2 063.5	2 341.7	2 320.4
Nori nei (*Porphyra* spp.)	424.9	703.1	1 040.7	1 109.9	1 312.9	1 733.1	2 017.8
Elkhorn sea moss (*Kappaphycus alvarezii*)	649.5	1 283.5	1 884.2	1 751.8	1 524.5	1 545.2	1 597.3
Brown seaweeds (Phaeophyceae)	2 852.8	1 827.2	3 021.2	436.8	805.0	666.6	891.5
Laver (*Porphyra tenera*)	529.2	584.2	565.2	688.5	713.4	831.2	855.0
Fusiform sargassum (*Sargassum fusiforme*)	12.1	115.6	97.0	209.3	216.4	254.6	268.7
Spiny eucheuma (*Eucheuma denticulatum*)	84.3	171.5	258.7	274.0	214.0	193.8	174.9
Spirulina nei (*Spirulina* spp.)	...	48.5	93.5	81.2	73.4	72.0	69.6
Seaweeds nei (algae)	32.5	13.6	8.9	15.2	15.8	20.0	22.5
Other algae	47.4	25.2	37.6	22.1	24.2	28.1	27.8
Total	**10 595.6**	**14 831.3**	**20 174.3**	**31 063.8**	**31 650.5**	**32 612.9**	**32 386.2**

[1] nei = not elsewhere included.
NOTE: … = no production, or production data unavailable.
SOURCE: FAO.

This growth in the demand for carrageenan accounts for approximately one third of the growth in total macroalgae production from 2000 to 2018 (Table 1). This rapid growth is not without consequence, as the practices used over the last 30 years have reduced the genetic variability of the commonly farmed seaweed strains and subsequently led to increased susceptibility to disease and pest infestation (Hurtado, Neish & Critchley 2019). Further, the practices used have had negative environmental effects such as bio-invasion of introduced cultivated seaweeds and overuse of non-environmentally friendly plastic ropes that are unable to be reused or recycled (Hurtado, Neish & Critchley 2019). Going forward, there needs to be increased regulation of farming expansions and processes, and further research to improve productivity, disease resistance and diversity of seedling supply (Hurtado, Neish & Critchley 2019).

The FAO recorded a total of 87 000 t of farmed microalgae in 2018, which is far below the approximate 32 million tonnes in live weight reported for macroalgae production (FAO 2020). This smaller representation of microalgae cultivation is in part due to underreporting owing to unavailable data from important producers including Australia

and the United States of America, possibly due to confidentiality. Despite this, the FAO acknowledge that farming of microalgae species such as S*pirulina* spp.,

Chlorella spp., *Haematococcus pluvialis* and *Nannochloropsis* spp. has been increasing due to the growing interest in their components for use as human nutrition supplements. These microalgae contain many potentially bioactive compounds including polyunsaturated fatty acids, peptides, polyphenols and carotenoids such as astaxanthin (Barkia, Saari & Manning 2019). The concentrations of these compounds can be increased by controlling growing conditions such as specific nutrient availability, light intensity, temperature and salinity (Barkia, Saari & Manning 2019; Zhu, Li & Hiltunen 2016). The ability to increase concentrations of these metabolites is critical, as they possess a number of health benefits including antihypertensive, anti-inflammatory and antioxidant effects (Barkia, Saari & Manning 2019). Due to the potential health benefits, the value of these bioactive compounds can greatly exceed that of the whole algal biomass (Table 2).

Table 2: Market value of selected high-value products isolated from microalgae compared to the whole biomass (Barkia, Saari & Manning 2019)

Product	Price (USD kg^{-1})	References
Astaxanthin	2500 - 7000	(Panis & Carreon 2016)
B-carotene	300 – 1500	(Borowitzka 2013)
Omega-3 fatty acids	80 – 160	(Borowitzka 2013)
Chlorella biomass	44	(Barkia, Saari & Manning 2019)
Arthrospira biomass	42 *	(Barkia, Saari & Manning 2019)

*Value expressed in € by the authors; the value shown was converted to USD ($1.17USD/€).

The growing global demand for nutritional supplements as well as functional foods and nutraceuticals with targeted health benefits has led to this high dollar value of algae bioactives and consequently the growth of the microalgae industry (Barkia, Saari & Manning 2019).

Although edible algae in a recognisable form is commonplace within typical Asian diets, macroalgae has not yet been widely consumed as part of a typical Australian diet. This is beginning to change, as the public focus is shifting towards using local food ingredients

and there is increasing recognition of the nutritional benefits of macroalgae (Mouritsen, Rhatigan & Pérez-Lloréns 2019). Further promotion of macroalgae or algal extracts for inclusion in the Australian diet would not only provide an additional nutritional source, but also could represent a solution to the demand for increased agricultural production, preferably sustainably, as a result of the growing global population (United Nations 2019).

Sustainable agriculture practices are designed to preserve the environment and available resources, whilst still being economically viable and producing sufficient human food, feed and fuel. The potential of this has already been realised in China, as seaweed agriculture is responsible for removal of 5.56% of nitrogen and 39.6% of phosphorus from the estimated total input to the Chinese coastal waters despite occupying only 0.3% of total Chinese territorial sea waters (Xiao et al. 2017). Eutrophication of Chinese coastal waters is a growing problem that has led to harmful algal blooms, such as the 2008 *Ulva prolifera* bloom that caused direct economic losses of an estimated $273 million AUD (Zhang et al. 2019). Chinese seaweed production has been growing at a rate of 7.96% per year, and although this rate of growth may not be sustainable, current seaweed agriculture is already delivering environmental and economic benefits to the country (Xiao et al. 2017).

One of the biggest factors for the sustainability of algae cultivation is the high photosynthetic efficiency and yields compared to terrestrial plants (Laurens 2017). These yields vary greatly depending on species and method of cultivation, however it is reported that these can be as high as ~55 tonnes per hectare per year (t ha^{-1} $year^{-1}$), roughly twice that of terrestrial plants (Laurens 2017). For comparison, one example would be soybean and a common macroalgae species in Australia called *Ulva ohnoi*. Soy protein is an important vegetable-sourced protein that is used for a variety of products including soy protein meat, protein powder and vegan dairy alternatives. Soybean has an average annual productivity of ~3 t ha^{-1} $year^{-1}$, resulting in a lipid productivity of 0.5 t ha^{-1} $year^{-1}$ and a protein productivity of 1.2 t ha^{-1} $year^{-1}$ (Ainsworth et al. 2012; Grieshop & Fahey 2001). In contrast, *U. ohnoi* has a lipid productivity of 2.8 t ha^{-1} $year^{-1}$, protein productivity of 18.4 t ha^{-1} $year^{-1}$ and in addition is a rich source of soluble fibre (16.4 t ha^{-1} $year^{-1}$) (Mata et al. 2016).

In general, algae far exceeds the yield of essential amino acids per hectare per year of other traditionally used protein sources and is able to achieve this whilst using almost negligible amounts of fresh water (Figure 1).

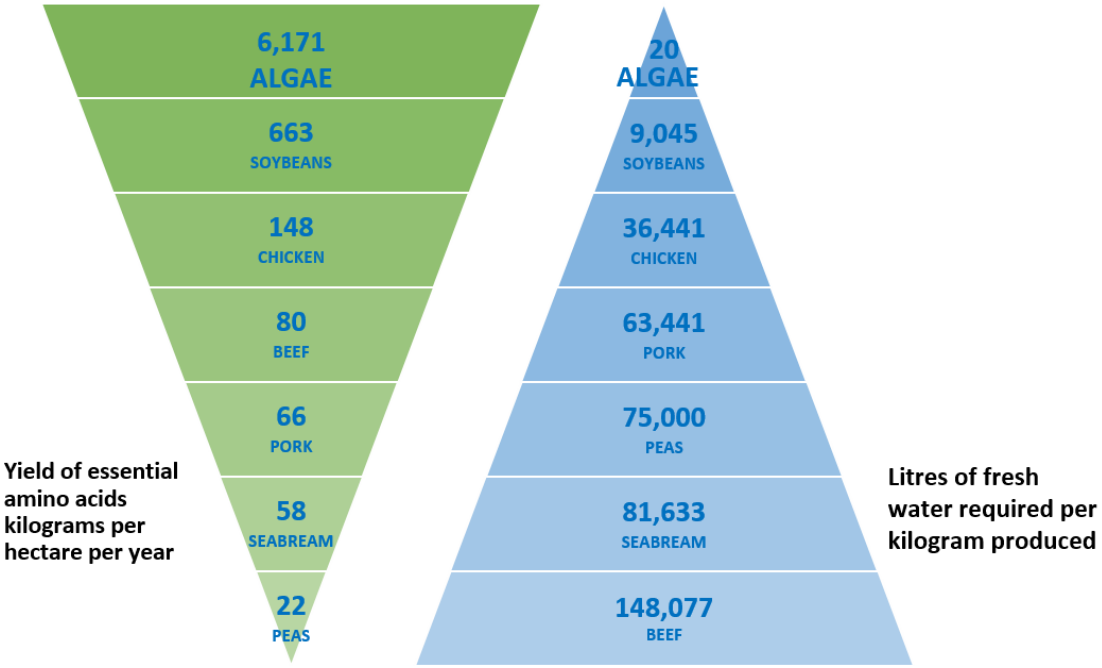


Figure 1: Yield of essential amino acids from common food sources and their associated water usage. Image courtesy of Dr Skye Thomas-Hall.

Marine algae cultivation does not require freshwater or arable land and thus does not need to compete with other crops for these resources. Further, algae have been successfully grown in a range of wastewater from sewage, manufacturing industries, aquaculture and agriculture (Arumugam et al. 2018). This has the dual benefit of both providing substrates for algal growth and removing organic and inorganic pollution from these wastewaters. Both macroalgae and microalgae have been successfully used for this purpose (Karray et al. 2017; Molazadeh et al. 2019). Many algae species have the ability to utilise and possibly accumulate nitrogen, phosphorus, carbon dioxide, heavy metals, pesticides, organic and inorganic toxins, and pathogens (Molazadeh et al. 2019). In doing so, they effectively remove these contaminants from wastewater to prepare the water for safe discharge into waterways.

Currently wastewater is commonly treated using microorganisms, primarily bacteria, to consume organic waste and other pollutants including nitrogen and phosphorus (Chan et al. 2009). The use of aerobic bacteria is advantageous due to their suitability for a range of environmental conditions and effectiveness in wastewater treatment; however, they require energy intensive aeration that greatly increases operating costs (Chan et al. 2009). Algae is a photosynthetic organism and consequently can produce oxygen to offset this cost-intensive aeration. Therefore, there is great potential for an algae-bacterial symbiotic system.

Studies using microalgae species such as *Chlorella sorokiniana* with common bacterial species has confirmed that growth of both bacteria and algae is increased in a symbiotic tank (Higgins et al. 2018; Ma et al. 2014). It was suggested that the algal production of sugars by photosynthesis stimulated bacterial growth, and that synthesis of vitamins from bacteria aided algal growth (Higgins et al. 2018), in addition to providing oxygen for bacterial growth and carbon dioxide for algal growth, as shown in Figure 2.

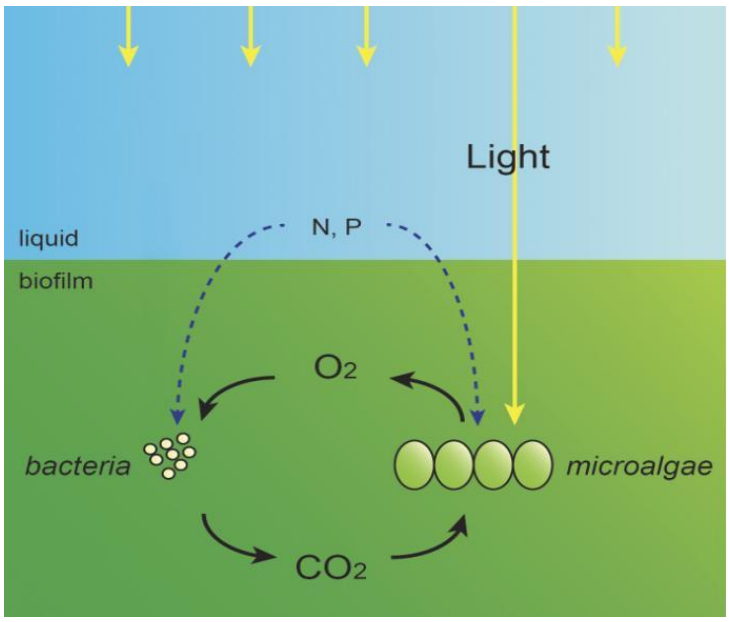


Figure 2: Schematic overview of the symbiotic algae-bacteria relationship. N; nitrogen, P; phosphorus (Boelee 2013).

Another study demonstrated that a co-culture of a microalgae, *Auxenochlorella protothecoides*, with *Escherichia coli* improved efficiency of oxidisable compound

degradation in wastewater and provided one-third of the biochemical oxygen demand by the bacteria (Holmes et al. 2020), hence improving the cost-effectiveness of wastewater treatment. The added benefit of this process is that sewage, agricultural waste and other underutilised food waste products like cheese whey wastewater provide nutrients for growth of the algal biomass that can then be further utilised (Molazadeh et al. 2019; Pandey, Srivastava & Kumar 2020; Wang et al. 2010).

Further utilisation of this algal biomass has already been explored for biofuel, particularly biodiesel, and bioenergy production (Zhou et al. 2012). The main sources used for fuel and energy production across the world are currently crude oil and coal, which are finite resources whose use has negative impacts on the ecological environment. Biodiesel is a form of fuel derived from the energy-rich oils contained in plants and animals and is consequently a renewable resource. Microalgae are a great source for biodiesel production as they naturally form a high oil content in relation to their dry weight (Adeniyi, Azimov & Burluka 2018). *A. protothecoides* used in wastewater treatment is also favourable for biodiesel production due to its high lipid accumulation rate (Zhou et al. 2012). Biodiesel production from algae is not yet financially feasible, so initiatives like this to use wastewater to cut down on costs could make this more of a reality (Doshi et al. 2017).

Organic waste from algae biomass can also be used to produce bioenergy through the conversion into biogas (Karray et al. 2017). Biogas is produced from the anaerobic degradation of organic matter and is predominantly a mixture of methane and carbon dioxide. This idea of generating multiple revenue streams from a single biomass can be further maximised by producing other bioproducts in a biorefinery facility. The biomass remaining after wastewater treatment, lipid extraction or bioenergy production can contain vitamins, proteins, polyunsaturated fatty acids, carotenoids and polysaccharides (Figure 3) (Campenni' et al. 2013; Neveux et al. 2015). These could be used for fertiliser, animal feed or human nutrition in either supplements or incorporated into food products (Neveux et al. 2015). This allows for full utilisation of the algae components and when combined with the high productivity and low resource requirements of algae advocates strongly for their potential as a sustainable crop with great economic potential.

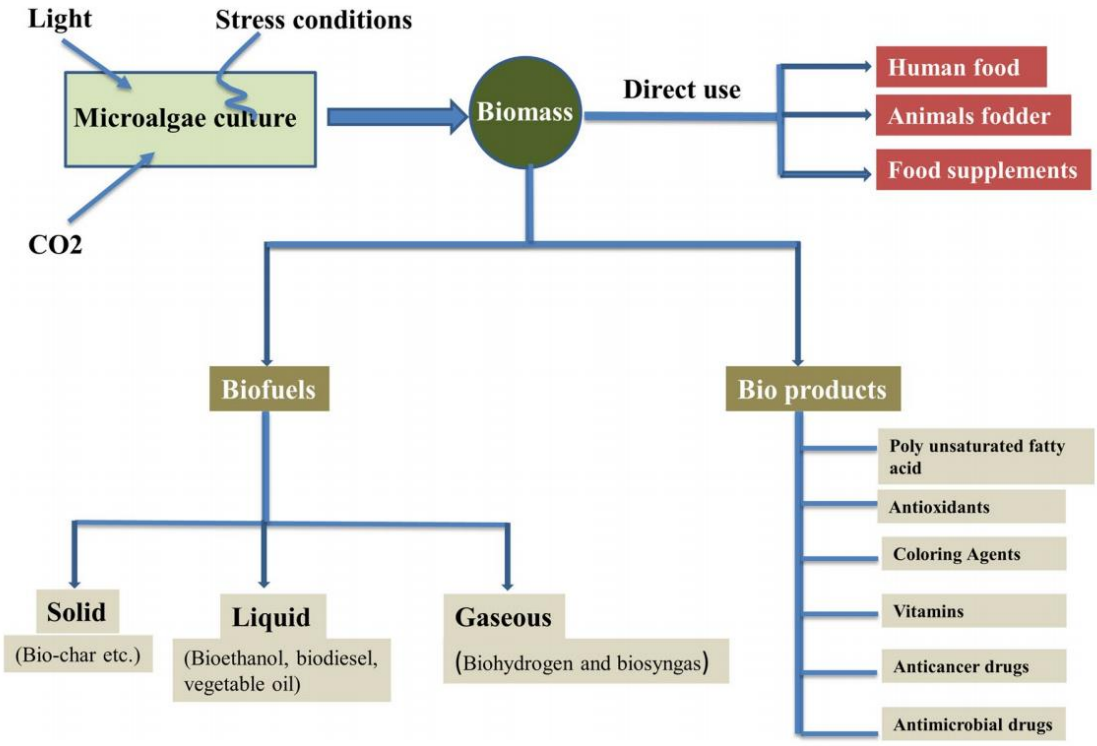


Figure 3: Microalgae biomass applications include biofuels, bio-products or direct use (Khan, Shin & Kim 2018)

However, minimal research has been undertaken to test and confirm the safety of wastewater-grown algae for human consumption. Further work is needed to assess the sensory acceptance of algae protein within human food products, but this is not a concern for the potential for algae use in animal feed. By using algae as a source of protein, carbohydrate and lipids in animal feed, they can be indirectly incorporated into the human food chain (Øverland, Mydland & Skrede 2019). Further, preliminary testing in dairy cows whose diet was supplemented with 1% *Asparagopsis armata* showed a 60% reduction in methane production through decreased rumen microbial production of methane (Roque et al. 2019). Livestock emissions contribute the majority of non-CO_2 greenhouse gas emissions, primarily methane, and this contribution was estimated to be responsible for 19% of total global warming that occurred in 2010 (Reisinger & Clark 2018). The Intergovernmental Panel on Climate Change governed by the United Nations has stated that to limit the predicted global warming to at least 1.5 °C there must be a 45% decrease in CO_2 emissions and a similar reduction in non-CO_2 emissions by 2030 (IPCC

2018). Otherwise, the climate-related risks to health, livelihoods, food security, water supply, human security and economic growth are predicted to worsen (IPCC 2018).

Additionally, extraction of algal products during biorefinery for application in cosmetics, nutritional and pharmaceutical industries is a possible high-value revenue stream. There are several promising algae components that have already been gaining interest for this purpose including carotenoids, polyphenols, sulphated polysaccharides and peptides as discussed earlier. Carotenoids are yellow, orange or red organic pigments that assist during light harvesting in photosynthesis and protect algae and plants from oxidative damage (Grossman, Lohr & Im 2004). This antioxidant property of carotenoids is of interest for human health, as it can potentially protect against reactive oxygen species (ROS)-induced disorders such as compromised immune response, certain cancers, obesity and diabetes (Fiedor & Burda 2014).

Carotenoids of interest for nutritional or pharmaceutical applications include astaxanthin, fucoxanthin, β-carotene and lutein (Gong & Bassi 2016). Polyphenols are another promising antioxidant algae component (Machu et al. 2015). Polyphenols are also found within fruits and vegetables, and their regular consumption from tea, grapes or extra virgin olive oil has been associated with reduced risk of cardiovascular disease, cancer and metabolic diseases (Gómez-Guzmán et al. 2018). Brown macroalgae contain a unique group of polyphenols known as phlorotannins. Phlorotannins may have antioxidant, blood pressure lowering and anticancer effects as well as protecting against the development of diabetes and chronic inflammation (Li, Y-X et al. 2011).

Some algal sulphated polysaccharides have already garnered commercial interest due to the application of carrageenan, agar and alginate as texturising agents in foods and beverages. However, there is growing interest in these sulphated polysaccharides as well as several others found in brown macroalgae, such as the fucoidans, and in green macroalgae, such as ulvan, for their potential health benefits (Wijesekara, Pangestuti & Kim 2011). Algae protein is already of interest for nutrition as a protein alternative to meat, as some species have potentially higher protein productivity than soybean, as discussed earlier. The discovery of short bioactive peptides of 2 – 20 amino acid residues has furthered this interest in algae protein, particularly the identification of angiotensin

converting enzyme (ACE) inhibiting peptides that lowered blood pressure in animal studies (Fan et al. 2014; Suetsuna, Maekawa & Chen 2004). Further work to understand the chemistry and potential health benefits of these algae components is necessary to determine their potential applications.

1.2 Chemistry of algae components

Algae contains a wide range of bioactive components including carotenoid pigments, sulphated polysaccharides, polyphenols, peptides, polyunsaturated fatty acids, vitamins and minerals. Whilst algae share some common compounds to terrestrial plants, such as many polyphenols and carotenoids, there remain a large number of bioactive compounds unique to algae (Michalak & Chojnacka 2015). The bioactive compounds common to both algae and other sources are still of interest due to the potential of algae to be used as a high yield, sustainable crop as discussed earlier.

The novel bioactive compounds are of great interest for pharmaceutical applications, in some cases due to their structural similarities to other well-characterised compounds with known health benefits (Cumashi et al. 2007). Astaxanthin and fucoxanthin are two unique carotenoid pigments produced by algae, which are of interest due to their structural similarities to antioxidant carotenoids such as β-carotene (Sathasivam & Ki 2018). There are many unique sulphated polysaccharides found within algae that are of interest for use both as texturising agents and as dietary fibre (Ngo & Kim 2013).

Phlorotannins are a group of polyphenols unique to brown algae that share structural similarities to the other tannin polyphenols found in terrestrial plants (Imbs & Zvyagintseva 2018). A growing number of algae peptides diverse in structure and function have been discovered in recent years. Although characterisation of these compounds is still ongoing, they represent further promising unique compounds produced by algae (Ovchinnikova 2019). It is reasonable to assume that the health benefits of these unique compounds will vary due to the differences in structure to other compounds within their respective classes. Therefore, it is important to first characterise and understand the structure of a compound before determining possible applications.

1.2.1 Carotenoids

There are over 750 carotenoids naturally synthesised by plants, algae, fungi and bacteria (Britton, Liaaen-Jensen & Pfander 2012). Carotenoids can absorb light in the visual range of the spectrum due to their structure, which generally contains a C_{40} hydrocarbon skeleton with a varying number of single and double bonds (Dutta, Chaudhuri & Chakraborty 2005). They are then classified as carotenes if the hydrocarbon chain does not contain oxygen, such as β-carotene (Figure 4) (Christaki et al. 2013). β-carotene is the most abundant carotene and is synthesised by plants, algae, fungi and bacteria (Britton, Liaaen-Jensen & Pfander 2012).

Carotenoids containing oxygen, either as a hydroxyl group, oxy-group or as a combination of both, are classified as xanthophylls (Christaki et al. 2013). Well-known examples of xanthophylls include lutein, astaxanthin and fucoxanthin (Figure 4). Lutein is synthesised by plants and algae, whereas astaxanthin and fucoxanthin are synthesised exclusively by algae. Whilst there are many other carotenoids found in algae, β-carotene, lutein, astaxanthin and fucoxanthin have all been widely studied for their promising health benefits so they will be the focus of this discussion (Sathasivam & Ki 2018).

The shared feature of these carotenoids and xanthophylls is the large number of conjugated double bonds present within their backbone (Dutta, Chaudhuri & Chakraborty 2005). Conjugated double bonds are alternating single and double bonds that enable the π orbital electrons to be shared within the conjugated system rather than being localised to one atom. This conjugated system consequently becomes a region of high electron density, and this is largely responsible for the pigment colour produced by the carotenoids as well as the antioxidant effect (Gammone, Riccioni & D'Orazio 2015). This is because the region of high electron density can quench singlet oxygen and thus prevent cellular damage (Dutta, Chaudhuri & Chakraborty 2005). Singlet oxygen is a strong oxidant that is produced in the body when ultraviolet (UV) radiation or visible light is absorbed by the body's natural pigments, such as melanin, and this energy is then transferred to molecular oxygen (Pospíšil, Prasad & Rác 2019).

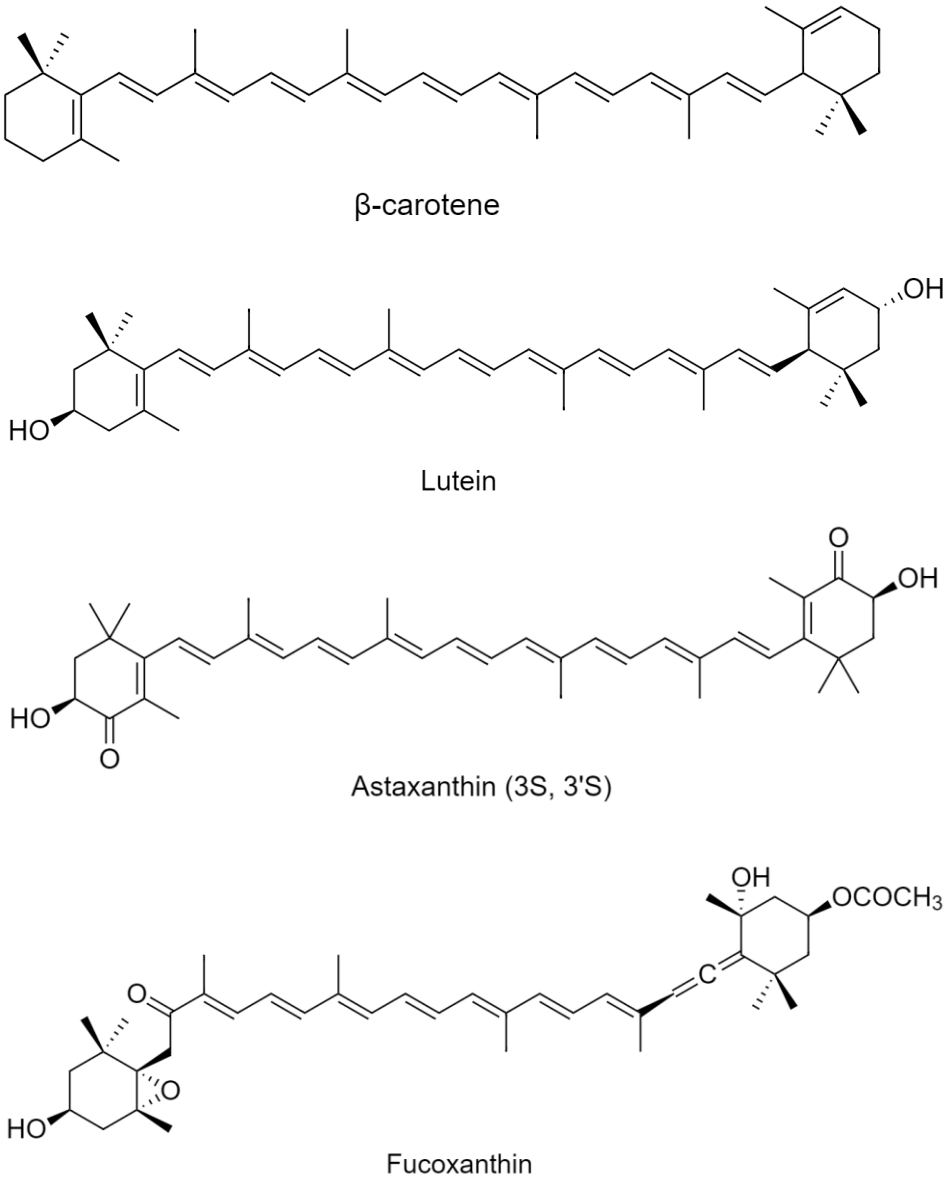


Figure 4: Major carotenoids of interest in algae

Inflammation is another source of singlet oxygen, mediated through phagocytes that release oxidising enzymes as part of their inflammatory response (Lambeth 2004). This transfer of energy changes the configuration of the oxygen π orbital to place two electrons within one π orbital that are moving in parallel directions, as shown in Figure 5. This configurational change increases the oxidising ability of singlet oxygen compared to molecular oxygen, thus enabling reaction with biomolecules to produce free radicals that

are highly reactive and can damage proteins, DNA and cell membranes (Pospíšil, Prasad & Rác 2019).

Superoxide radical anion is an example of a free radical that contains one unpaired electron and consequently is highly reactive to take an electron from other compounds to attain stability (Figure 5) (Phaniendra, Jestadi & Periyasamy 2015). Carotenoids can also react with these free radicals to stabilise their structure and this is known as free radical scavenging (Dutta, Chaudhuri & Chakraborty 2005).

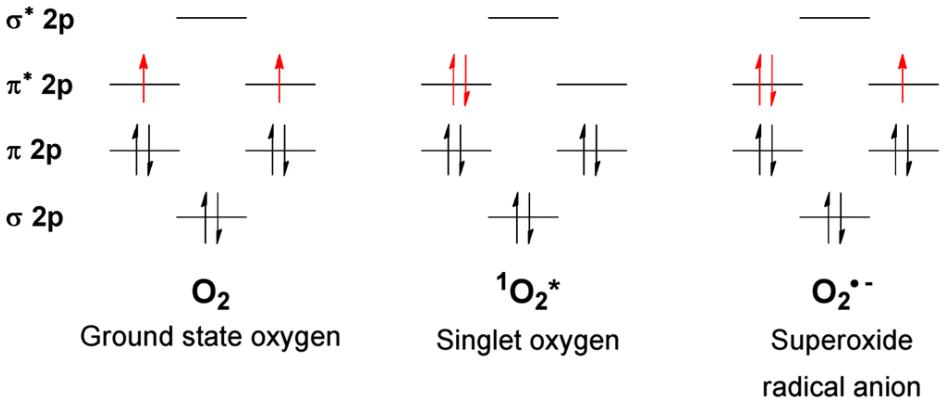


Figure 5: Molecular orbital diagrams for ground-state molecular oxygen (O_2), singlet oxygen ($^1O_2$), and superoxide radical anion O_2^- *(Krumova & Cosa 2016)*

Differences in the potency of singlet oxygen quenching and free radical scavenging between carotenoids are due to their variations in structure (Dutta, Chaudhuri & Chakraborty 2005). More double bonds increase the efficacy of the conjugated double bonds in singlet oxygen quenching (Stahl & Sies 2003). The presence of hydroxyl (OH), epoxy (oxygen atom joined to two adjacent carbon atoms) and methoxy (OCH_3) groups appears to lower antioxidant potency (Sachindra et al. 2007). However, a hydroxyl group adjacent to a carbonyl group (carbon oxygen double bond) enables the formation of a stable complex with metal ions (Brotosudarmo, Limantara & Setiyono 2020). This indirectly prevents oxidation and cellular damage, as metals ions such as Fe^{2+} are catalysts in the formation of hydroxyl radicals (Brotosudarmo, Limantara & Setiyono 2020). For

these reasons, the antioxidant properties of astaxanthin are reported to be up to 10 times more potent than β-carotene and lutein (Miki 1991; Naguib 2000).

Astaxanthin contains two carbonyl groups adjacent to hydroxyl groups and 11 conjugated double bonds (Figure 4). Due to the polar hydroxyl groups in the structure of astaxanthin, it is also able to be positioned across the cellular membrane rather than be confined to the hydrophobic interior as is β-carotene (Figure 6). This positioning enables astaxanthin to protect the lipid membrane from oxidation (Jomova & Valko 2013). Astaxanthin is a red lipophilic pigment that is approved for consumption as a food colourant in animal and fish feed by the United States Food and Drug Administration (Roche 1987). The richest source of astaxanthin is the microalgae *Haematococcus pluvialis* as 81.2% of the carotenoid content of 2-5% in the red stage or up to 3.8% on dry weight (Shah et al. 2016). It is also found in marine animals such as salmon due to their diet (Ambati et al. 2014).

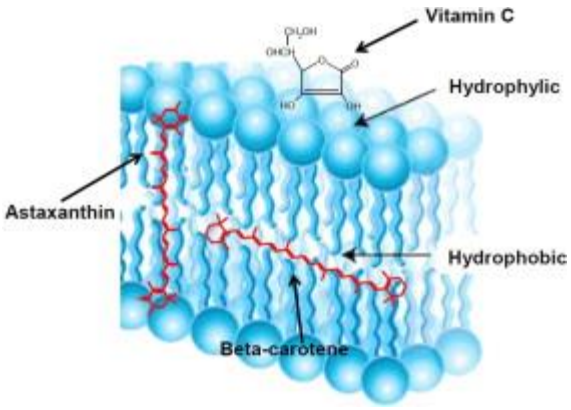


Figure 6: The localization of carotenoids in biological membranes (Jomova & Valko 2013)

β-carotene and lutein contain 10 conjugated double bonds and have long been recognised for their antioxidant potency (Moeller et al. 2008; Naguib 2000). β-carotene is a red-orange lipophilic pigment that is converted into two molecules of retinal in the body and has the highest provitamin A activity of all the carotenoids (Green & Fascetti 2016). Algal sources of β-carotene include *Spirulina* spp., *Chlorella* spp., and *Dunaliella* spp.

(Sathasivam & Ki 2018). Lutein is a yellow lipophilic pigment that is found within *Chlorella* spp., *Scenedesmus* spp. and *Dunaliella* spp. (Sun et al. 2015).

Fucoxanthin is a brown pigment that is found in a range of macroalgae and microalgae including *Undaria pinnatifida, Phaeodactylum tricornutum* and *Odentella aurita* (Zhang et al. 2015). Fucoxanthin has not been studied as extensively as these carotenoids but it has been reported to be a less potent antioxidant (Sachindra et al. 2007). It contains 7 conjugated double bonds and also contains hydroxyl and epoxy groups, and this is thought to explain why the singlet oxygen quenching rate of β-carotene was approximately 10 times faster than fucoxanthin (Sachindra et al. 2007), although the capacity for radical scavenging by fucoxanthin appears to be higher than β-carotene and comparable to that of lutein (Rodrigues, Mariutti & Mercadante 2012). Fucoxanthin has reported anti-obesity effects and this could be due to its unique structure compared to the other carotenoids (Zhang et al. 2015). However, more research is needed to confirm these anti-obesity effects.

It is well-recognised that the concentration of these carotenoids can be elevated by placing the algae under stress conditions such as high salinity, nitrogen deficiency, high temperature or high light intensity (Sarada, Tripathi & Ravishankar 2002; Xie et al. 2020). A study comparing nitrate restriction and a variety of LED intensities and colours, reported that low nitrate and either green LED or warm white LED exposure for 23 days increased the carotenoid concentration in *H. pluvialias* by 108 and 101-fold respectively (Saha et al. 2013). This technique is beneficial for optimising yields in commercial production of carotenoids.

1.2.2 Sulphated Polysaccharides

Algae are a rich source of sulphated polysaccharides, which are long-chain carbohydrates comprised of repeating monosaccharide units with sulphate esters joined together by glycosidic bonds (Figure 7). These sulphated polysaccharides are located within the algal cell wall and the type differs between red, brown and green macroalgae. The major algal sulphated polysaccharides are carrageenans in red macroalgae, fucoidans in brown macroalgae and ulvans in green macroalgae (Patil et al. 2018).

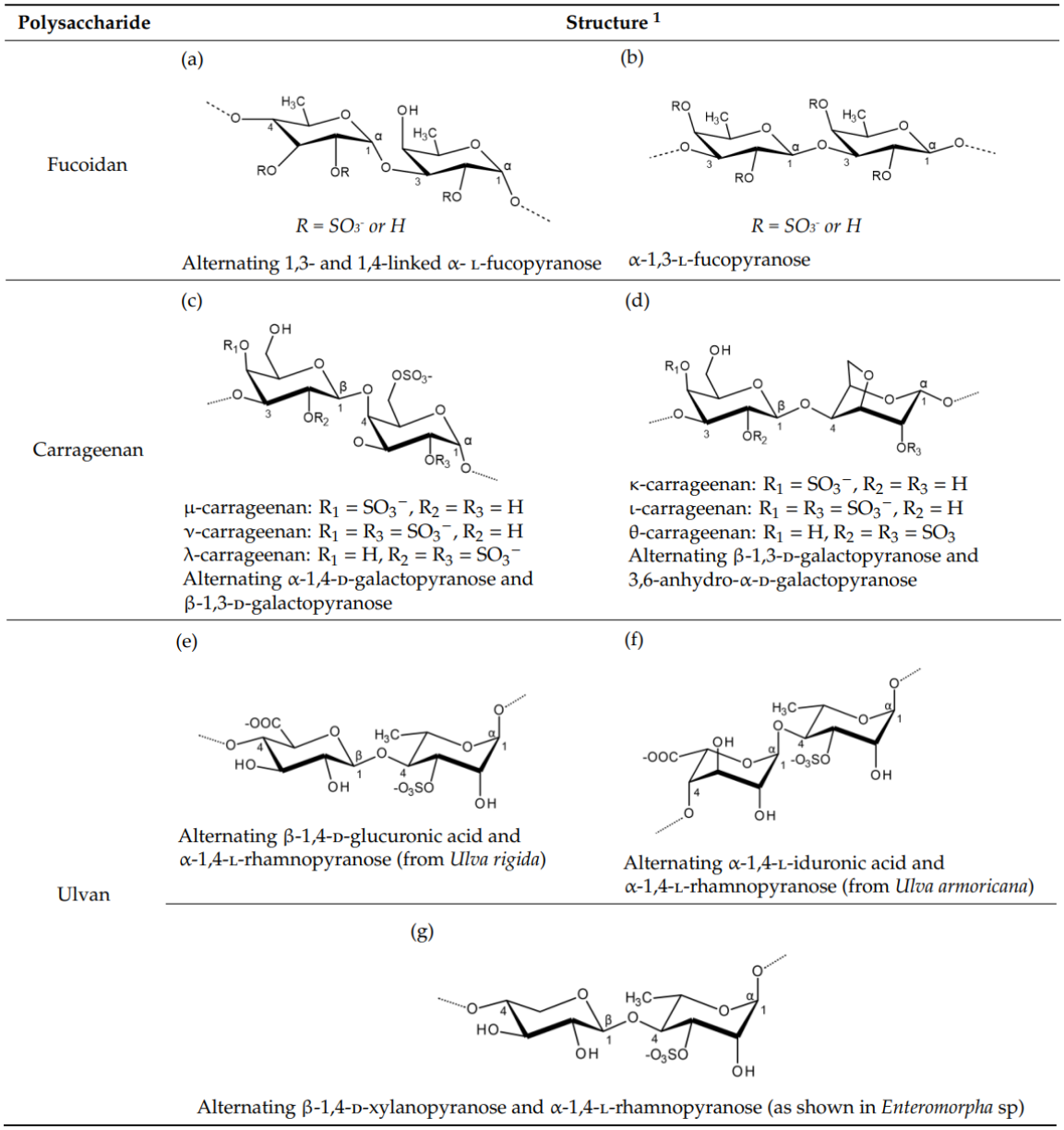


Figure 7: Basic structures of fucoidans, carrageenans and ulvans (Jönsson et al. 2020)

These sulphated polysaccharides vary in their constituent monosaccharide units, type of glycosidic bond, degree of sulphation, degree of branching, molecular mass and conformation. There is further variation of the structure and concentration within each sulphated polysaccharide depending on the species of algae, environmental conditions, time of harvest and the extraction method (Jönsson et al. 2020).

Despite these differences, there are still similarities in structure and consequently in function. Algal sulphated polysaccharides are water-soluble and resist hydrolysis by digestive enzymes in the small intestine of humans (Cunha & Grenha 2016). These characteristics define them as soluble fibre. Soluble fibre attracts and binds water and, depending on the structure, can subsequently form a viscous gel (Chutkan et al. 2012). The formation of this viscous gel is beneficial for commercial applications, as it allows sulphated polysaccharides to be used as gelifying, stabilising or emulsifying agents in food (Cunha & Grenha 2016).

Structural features that enhance solubility include a branching structure, the presence of ionic groups such as sulphate or hydroxyl, and variable glycosidic bonds and monosaccharide units. Linear chains, high molecular weights and regular conformations lower solubility, as they enhance the intermolecular association of the sulphated polysaccharides (Goff & Guo 2019). The viscosity of the soluble fibre increases as the molecular weight or chain length increases (Dhingra et al. 2012). The molecular weight and the degree of sulphation can be partially determined through the extraction method used and this allows producers to alter the sulphated polysaccharide product after algal growth (Kidgell et al. 2019).

Carrageenans are a linear chain of repeating D-galactose units in the pyranose form. Pyranose form refers to a six-membered cyclic ring, containing five carbons and one oxygen atom. There are at least 15 different carrageenan structures, which as shown in Figure 7c-d primarily differ in their number and position of sulphate groups and also the presence of 3,6-anhydro-α-D-galactopyranose (3,6-AG) (Jiao et al. 2011). The 3,6-AG configuration is characterised by the formation of a ring across the 3 and 6 positions as shown in Figure 7d and is hydrophobic in nature (Cunha & Grenha 2016).

Three commonly occurring carrageenan structure types are kappa (κ-carrageenan), iota (ι-carrageenan) and lambda (λ-carrageenan) (Necas & Bartosikova 2013). κ-carrageenan and ι-carrageenan are quite similar in their solubility and viscosity as they have an ester sulphate content of 25 – 30% and 28 – 35% respectively and both contain approximately 30% 3,6-AG, whereas λ-carrageenan is highly sulphated, about 32 – 39%, and does not contain 3,6-AG. λ-carrageenan is therefore highly soluble due to the high sulphate content

and absence of 3,6-AG. However, λ-carrageenan cannot form a gel due to the high number of sulphate esters preventing formation of a helical structure (Cunha & Grenha 2016). Both κ-carrageenan and ι-carrageenan are able to form gels, particularly κ-carrageenan due to the lowest sulphate content (Cunha & Grenha 2016). The average molecular weight of carrageenan is above 100 kDa (Necas & Bartosikova 2013).

Unlike carrageenans, which are well characterised and consistently contain galactose, fucoidans are highly variable in structure as they can contain a wide range of monosaccharides, uronic acids, acetyl groups and proteins (Li et al. 2008). The molecular weight can also vary greatly, ranging from 10 kDa to 2000 kDa (Citkowska, Szekalska & Winnicka 2019). This fluctuation in structure is predominantly between different brown macroalgae species, however there are still some common features (Van Weelden et al. 2019). Fucoidans are generally a highly branched chain with a backbone of repeating L-fucose units in the pyranose form (Figure 7a-b) (Cunha & Grenha 2016). The sulphate esters are frequently attached to the C-2 or C-4 position and in general the sulphation grade ranges from 4 to 8% (Cunha & Grenha 2016). Due to the high level of branching and moderate sulphation, fucoidans are highly water-soluble but they do not form a gel. Consequently, fucoidans are not used commercially as a texturising agent and interest in this polysaccharide group is due to promising pharmaceutical applications such as anti-cancer effects (Senthilkumar et al. 2013). It is difficult to comment further on the structure of fucoidans due to the high variability even within the same algal species. Fucoidans are widely found in brown macroalgae, including rich sources such as *Sargassum* spp., *Undaria pinnatifida* and *Fucus vesiculosus* (Cunha & Grenha 2016).

The structure of ulvans is also highly variable but it is far less studied than either carrageenans or fucoidans (Cunha & Grenha 2016). In contrast to the consistent monosaccharide unit that is generally found in both carrageenans and fucoidans, ulvans are instead comprised of repeating disaccharide units known as either ulvanobiouronic acids or ulvanobioses (Lahaye & Robic 2007). Ulvanobiouronic acids are shown in Figure 7e-f and are comprised of either a glucuronic acid or iduronic acid bound to a L-rhamnose unit. An example of an ulvanobiose is shown in Figure 7g and consists of a D-xylose joined to a L-rhamnose unit. The D-xylose of the other ulvanobiose type is sulphated (Kidgell et

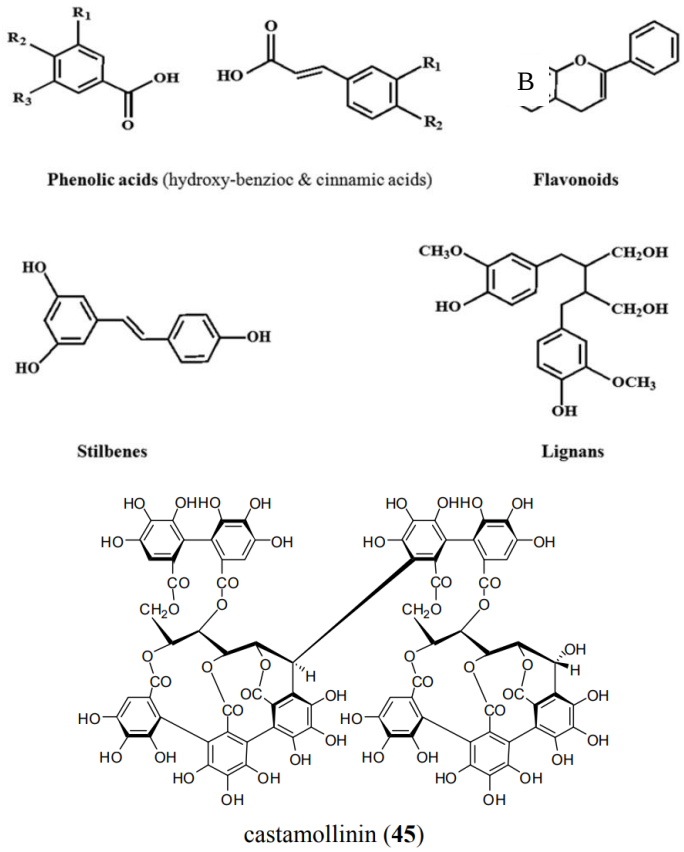
al. 2019). The ulvanobiouronic acids occur more commonly than the ulvanobioses (Kidgell et al. 2019). Rhamnose is generally the predominant component of ulvans as it forms a part of both ulvanobiouronic acids and ulvanobioses, and of the *Ulva* spp. studied so far it accounts for an average 45 mol% (Kidgell et al. 2019). However, the amount of rhamnose varies greatly from 5 – 92.2 mol% and glucose can predominate in some species accounting for up to 87 mol% (Kidgell et al. 2019).

It is not yet understood how other monosaccharides such as glucose are arranged within the ulvan structure, although the ulvan backbone is lightly branching (Kidgell et al. 2019; Robic et al. 2009). Different features of the ulvan structure compared to carrageenans and fucoidans are that the rhamnose contains a methyl group and the glucuronic acid and iduronic acid contain a carboxylate group (Figure 7). While ulvans should be highly water-soluble due to their hydrophilic ionic groups, they are instead only poorly soluble and this is thought to be due to the methyl group (Robic et al. 2009). This methyl group also contributes to the bead-like aggregates that form when ulvans are in solution, which cause ulvans to have low viscosity (Robic et al. 2009). There is the potential for ulvans to form gels, however this is only in the presence of borate and divalent cations such as calcium ions within a controlled pH solution (Kidgell et al. 2019).

1.2.3 Polyphenols

The group of compounds known as polyphenols includes over 8000 different compounds, which are classified according to the number of phenol rings and the other structural elements that bind these rings together (Pandey & Rizvi 2009). Phenol rings are benzene rings (6-carbon structure) that have an attached hydroxyl group. Polyphenols are divided into five different classes as flavonoids, phenolic acids, stilbenes, lignans and tannins (Figure 8) (Pandey & Rizvi 2009).

A



B

Figure 8: A; chemical structures of the different polyphenols (Pandey & Rizvi 2009). B; example of ellagitannin dimer (Yoshida, Amakura & Yoshimura 2010)

The basic skeleton of the flavonoids contains two benzene rings linked by a nonaromatic ring containing five carbon and one oxygen atoms (Panche, Diwan & Chandra 2016). The flavonoids are the most diverse group of the polyphenols, accounting for over 6000 of the 8000 different polyphenols, and are divided into six subgroups (Panche, Diwan & Chandra 2016). The flavonoid subgroups are identified by the carbon number of the ring attachment site and the degree of unsaturation and oxidation (Panche, Diwan & Chandra 2016). Phenolic acids are derived from either cinnamic acid (hydroxycinnamic acids) or benzoic acid (hydroxybenzoic acids) (Kumar & Goel 2019). Stilbenes and lignans are found almost exclusively in terrestrial plants (Peterson et al. 2010; Rivière, Pawlus & Merillon 2012). The tannins are differentiated from the other polyphenol classes by their relatively high molecular weight and the ability to associate with carbohydrates and proteins to form polymers (Fraga-Corral et al. 2020). The tannins are divided into gallotannins,

ellagitannins (castamollinin example shown in Figure 8), complex tannins, condensed tannins and phlorotannins. The phlorotannins are unique to brown algae and the other tannins are widely distributed in land and marine plants (Fraga-Corral et al. 2020).

A compilation of phenolic acids, flavonoids and phlorotannins identified in algae is shown in Table 3. It can be observed from the table that there is considerable overlap in polyphenol content across green, brown and red algae species. This prevalence across many plant sources, as well as the potential for antioxidant and anti-inflammatory effects, has generated a large degree of interest in these compounds (Zhang & Tsao 2016). The antioxidant activity is attributed to the aromatic groups, highly conjugated system (as in carotenoids) and multiple hydroxyl groups (Zhang & Tsao 2016). However, the anti-inflammatory structure-function relationship is still not well understood (Ambriz-Pérez et al. 2016). As the phlorotannins are unique to algae, further discussion will focus on this class of polyphenols.

Table 3: A list of phenolic compounds isolated from marine algae (Fernando et al. 2016)

	Seaweed	*Identified compounds*
Phenolic acids	*Halimeda monile*	Salicylic, gallic, and caffeic acids
	Laminaria digitata	Gallic, protocatechuic, gentisic hydroxybenzoic, chlorogenic, vanillic, caffeic
	Dictyota dichotoma	Gallic, protocatechuic, gentisic, *p*-hydroxybenzoic, chlorogenic, vanillic, syringic
	Fucus vesiculosus	Gallic, protocatechuic, gentisic, chlorogenic, vanillic, caffeic
	Fucus serratus	Gallic, protocatechuic, gentisic, chlorogenic, vanillic, caffeic
	Fucus distichus	Gallic, protocatechuic, gentisic, chlorogenic, caffeic
	Fucus spiralis	Gallic, protocatechuic, gentisic, chlorogenic, vanillic, caffeic
	Mastocarpus stellatus	Gallic, protocatechuic, gentisic, *p*-hydroxybenzoic, chlorogenic, vanillic, caffeic
	Polysiphonia fucoides	Gallic, protocatechuic, gentisic, chlorogenic, syringic, caffeic, salicylic
	Saccharina latissima	Gallic, protocatechuic, gentisic, *p*-hydroxybenzoic, chlorogenic, vanillic, syringic
	Gracilaria vermiculophylla	Gallic, protocatechuic, gentisic, *p*-hydroxybenzoic, vanillic, syringic, salicylic
	Palmaria palmata	Gallic, protocatechuic, gentisic, *p*-hydroxybenzoic, chlorogenic, syringic, caffeic
	Porphyra purpurea	Gallic, protocatechuic, gentisic, *p*-hydroxybenzoic, chlorogenic
	Chondrus crispus	Gallic, protocatechuic, gentisic, *p*-hydroxybenzoic, chlorogenic, syringic, caffeic, ferulic
	Ulva lactuca	Gallic, protocatechuic, gentisic, *p*-hydroxybenzoic, chlorogenic, vanillic, syringic, caffeic, salicylic, coumaric
	Sargassum muticum	Gallic, protocatechuic, gentisic, *p*-hydroxybenzoic, chlorogenic, vanillic, syringic, caffeic
	Enteromorpha intestinalis	Gallic, protocatechuic, gentisic, *p*-hydroxybenzoic, chlorogenic, vanillic, syringic, caffeic, salicylic
	Spongiochloris spongiosa	Protocatechuic, *p*-hydroxybenzoic, 2,3-dihydroxybenzoic, chlorogenic, vanillic, caffeic, *p*-coumaric, salicylic acid, and cinnamic acid
	Anabaena doliolum	Protocatechuic, *p*-hydroxybenzoic, 2,3-dihydroxybenzoic, chlorogenic, caffeic, *p*-coumaric, salicylic acid, and cinnamic acid
	Spirogyra sp.	Gallic acid, methyl gallate
Flavonoids	*Acanthophora spicifera*	Acanthophorin A, acanthophorin B, tiliroside, catechin, quercetin
	S. muticum, *Hypnea spinella*, *Porphyra* sp., *C. crispus*, *Halopytis incurvus*	Daidzin, genistin, ononin, daidzein, sissotrin, genistein, formononetin, biochanin
	Undaria pinnatifida	Daidzin, genistin, ononin, sissotrin, formononetin biochanin
	Acetabularia ryukyuensis	Catechin, epicatechin
	Tydemaniz expeditionis, *Caulerpa serrulata*	Catechin, epigallocatechin
	Caulerpa racemosa	Epigallocatechin
	Eisenia bicyclis	Catechin, epicatechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate
	Hizikia fusiformis	Epigallocatechin
	Laminaria religiosa, *Sargassaceae ringgoldianum*	Catechin
	Ishige okamurae	Catechin, epigallocatechin
	Padina arborescens	Catechin, epigallocatechin, epigallocatechin gallate
	Padina minor	Catechin, epicatechin, epigallocatechin gallate
	Hydroclathrus clathratus	Catechin
	Sargassum thunbergii	Catechin, epigallocatechin
	S. muticum	Epicatechin, epigallocatechin, epicatechin gallate
	Turbinaria ornata	Catechin, epigallocatechin
	Porphyra yezoensis	Catechin, epigallocatechin gallate
	Chondrus verrucosus, *Ceratodictyon spongiosum*	Catechin
	Gloiopeltis complanata	Catechin, epigallocatechin
	Gelidium elegans	Catechin, epicatechin, catechin gallate
	Chondrococcus hornemannii	Catechin, epicatechin, epigallocatechin, epigallocatechin gallate

	Gracilaria texorii, *Gracukarua asiatica*	Epigallocatechin, epigallocatechin gallate
	Caulerpa sertularioides	Gallocatechin, epicatechin, catechin gallate
	Turbinaria conoides	Epigallocatechin, catechin, epicatechin
	Sargassum polycystum	Epigallocatechin gallate
	Padina australis	Catechin
Phlorotannins	*Ecklonia cava*	Phloroglucinol, eckol, phlorofucofuroeckol A, dieckol and 8,8′-bieckol, 6,6′-bieckol, dioxinodehydroeckol
	E. bicyclis and Ecklonia Kurome	Phloroglucinol, eckol, phlorofucofuroeckol A, dieckol and 8,8′-bieckol
	Ecklonia stolonifera	Phlorofucofuroeckol A, dieckol, dioxinodehydroeckol, eckstolonol, triphlorethol-A, fucosterol, phloroglucinol, eckol, phlorofucofuroeckol-A, 2-phloroeckol, 7-phloroeckol
	F. distichus	A trimeric fucol, phloroethol, fucophloroethol
	Eisenia arborea	Phlorofucofuroeckol-B
	I. okamurae	Diphlorethohydroxycarmalol
	Ishige foliacea, *Ishige sinicola*	Octaphlorethol A
	Cystophora congesta	Phloroglucinol triacetate, diphlorethol pentaacetate, bromotriphlorethol-A1-heptaacetate, bromotriphlorethol-A2-heptaacetate, tetraphlorethol-C-nonaacetate, fucodiphlorethol-D-decaacetate, triphlorethol-A-heptaacetate
	Analipus japonicus	Difucol hexaacetate, trifucol nonaacetate, and tetrafucol-A and B dodecaacetates

Phlorotannins are located primarily within the cytoplasm of brown algae, although they also form complexes with components of the cell wall and thus impact structural development (Heffernan et al. 2015). They function to defend the algae against herbivores, microbes and UV light damage, and consequently their concentration can be potentially increased through control of commercial production (Catarino, Silva & Cardoso 2017). Early investigations into this possibility has shown that introducing sea snails (herbivore) to growing *Ascophyllum nodosum* increased phlorotannin concentration by 23% and UVA and UVB radiation enlarged the size and number of phlorotannin-containing vesicles in two species of kelp (*Alaria esculenta* and *Saccorhiza dermatodea*) (Pavia & Brock 2000; Wiencke, Clayton & Schoenwaelder 2004).

The total concentration of phlorotannins in brown algae can be up to 25% of dry weight, and this amount naturally increases in response to environmental increases in light exposure and salinity (Connan & Stengel 2011; Ford et al. 2019). Phlorotannins are comprised of monomeric units of phloroglucinol, shown in Figure 9, which are connected together by either C-C or C-O-C bonds to form polymers (Ford et al. 2019). These polymers are structurally diverse in number of phloroglucinol units, up to a total of 16 with varying linkage position and bond type. Antioxidant capacity of phlorotannins tends to decrease with increasing polymerisation, perhaps due to configuration changes that

enclose the hydroxyl groups making them unavailable for antioxidant activity (Hermund et al. 2018).

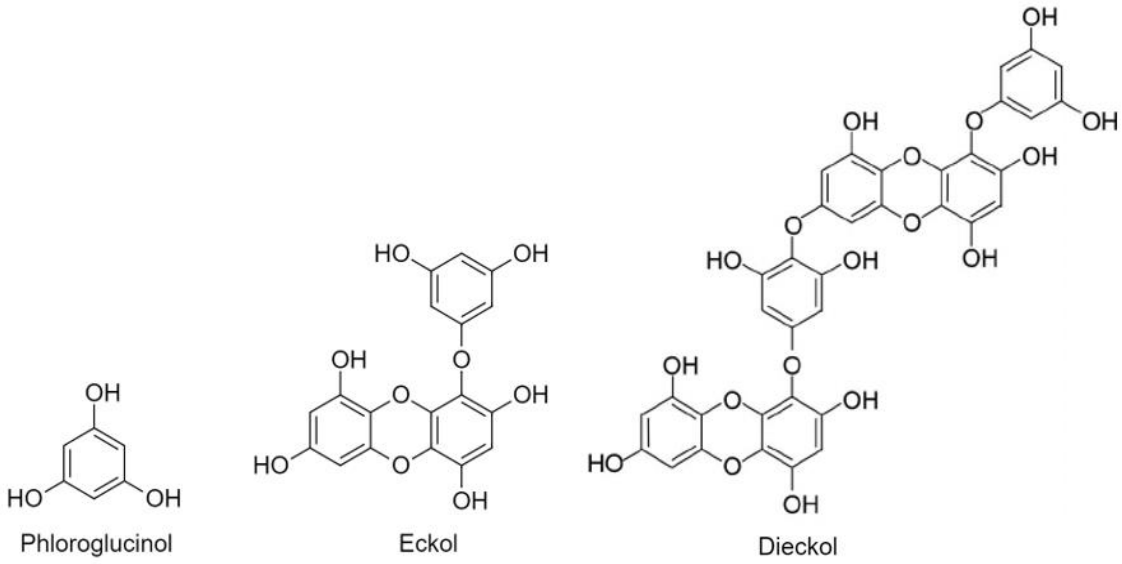


Figure 9: Monomeric unit of phlorotannins (phloroglucinol) and eckol and dieckol as examples of phlorotannin polymers

Eckol is a trimeric polymer found in *Ecklonia cava,* as well as in other brown algae species, that has a relatively low molecular weight of 372 g/mol whilst still containing six hydroxyl groups (Figure 9). This compound showed higher radical scavenging activity and more effective inhibition of DNA damage from oxidation than either phloroglucinol or dieckol (molecular weight 742 g/mol) (Ahn et al. 2007). Further, eckol displayed greater ACE-inhibition than phloroglucinol or dieckol (Jung et al. 2006) and there is potential for anti-inflammatory, anti-diabetic and anti-coagulative activity (Manandhar et al. 2019). Further testing in animal and *in vitro* and characterisation of phlorotannin structures is necessary to guide understanding of phlorotannin structure-function relationships.

1.2.4 Peptides

Bioactive peptides are short protein fragments that contain between 2 – 20 amino acids and originate from a larger parent protein found in animal and plant products (Fan et al. 2014). The bioactive peptide is inactive whilst contained within the larger protein

molecule, and is released and thus activated by enzymatic cleavage during digestion or food processing (Sánchez & Vázquez 2017). To be considered bioactive, the peptide must then be able to exert a biological effect on the body, either through crossing the intestinal barrier into the blood circulation or exerting local effects in the gastrointestinal tract (Manikkam et al. 2016). The subsequent biological effect in the body is dependent on the amino acid composition and sequence, structural conformation, and ability to pass through the intestinal barrier (Manikkam et al. 2016). However, it is not currently known how to predict the biological effect based on the chemical structure (Sánchez & Vázquez 2017). Despite this, the chemical structure and function of multiple bioactive peptides from algae have been characterised. Before these will be discussed, it is important to first define the structure of amino acids and peptide chains.

An amino acid contains a central carbon atom, an amino group (NH_2), a carboxyl group (COOH) and a unique side chain (R). The unique side chain can be comprised of either a singular atom or group of atoms, and this determines the size, polarity and pK of the amino acid (Bischoff & Schlüter 2012). There are 22 naturally occurring proteinogenic amino acids (Bischoff & Schlüter 2012). When they are linked together to form a peptide, a molecule of water is released and a peptide bond forms between the carbon of the carboxyl group of one amino acid and the nitrogen of the amino group of another amino acid (Figure 10).

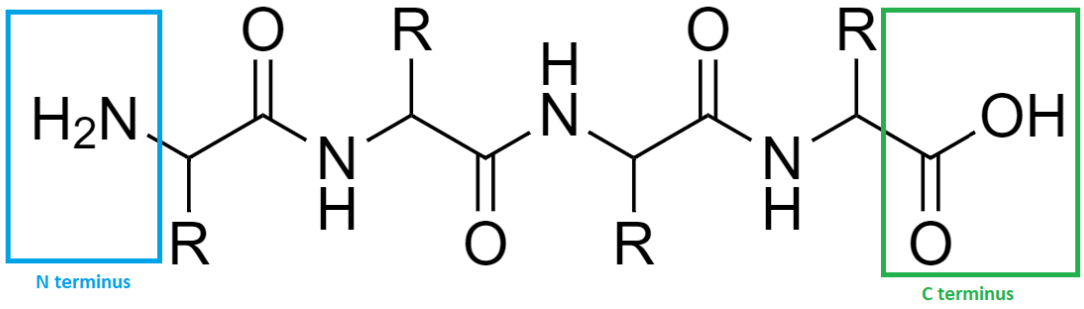


Figure 10: Peptide chain structure

This consequently leaves the first amino acid with an unaltered amino group, defined as the N terminus, and one amino acid with an unaltered carboxyl group, defined as the C

terminus. The peptide amino acid sequences discussed in this review will follow this order, starting with the C terminal and ending in the N terminal amino acid. The bioactive peptides with antihypertensive and antioxidant effects will be the focus of this discussion.

Table 4 lists the promising antihypertensive bioactive peptides and their parent protein and algal source. Both the enzymes ACE and renin are recognised to be prohypertensive and thus inhibition of these enzymes is antihypertensive (Fan et al. 2014). The sequence and composition of the amino acids in these peptides appears to be highly variable, however there are some common structural features.

Table 4: Algae-derived antihypertensive bioactive peptides (Fan et al. 2014)

peptide name or sequence	source	enzyme	IC_{50}	in vitro/in vivo	mechanism of action
YH, KY, FY, IY	*Undaria pinnatifida*	no enzyme used	2.7−43.7 μmol/L	in vitro and in vivo (rats)	ACE inhibition
enzymatic digests	*Ecklonia cava*	Kojizyme, Flavourzyme, Neutrase, Alcalase, and Protamex	2.33−3.56 μg/mL	in vitro	ACE inhibition
VECYGPNRPQF	*Chlorella vulgaris*	pepsin	29.6 μM	in vitro	ACE inhibition
VEGY	*Chlorella ellipsoidea*	Protamex, Kojizyme, Neutrase, Flavourzyme, Alcalase, trypsin, α-chymotrypsin, pepsin, and papain	128.4 μM	in vitro and in vivo (rats)	ACE inhibition
GMNNLTP, LEQ	*Nannochloropsis oculata*	pepsin, trypsin, α-chymotrypsin, and papain	123−173 μM	in vitro	ACE inhibition
IRLIIVLMPILMA	*Palmaria palmata*	papain	3.3 mM	in vitro	renin inhibition

The bioactive peptides that can bind directly to the active site of the ACE enzyme generally contain a branched chain aliphatic amino acid at the N-terminal and either tryptophan (W), tyrosine (Y), proline (P) or phenylalanine (F) at their C-terminal (Manikkam et al. 2016). The branched chain aliphatic amino acids include valine (V), leucine (L) and isoleucine (I) (White & Newgard 2019). The active site of ACE also cannot accommodate large peptide molecules (Manikkam et al. 2016). Bioactive peptides sourced from *Undaria pinnatifida*, *Chlorella* vulgaris and *Chlorella ellipsoidea* adhere to this restriction and thus are likely to be competitive ACE inhibitors, although this mechanism has not yet been confirmed (Fan et al. 2014). The other bioactive peptides listed in Table 4 have still reported ACE inhibitory effects, and this could be due to non-competitive inhibitory mechanisms so further research is required to understand the

chemical structure-function relationship. In general, it is recognised that hydrophilic peptides possess minimal ACE inhibitory effects (Manikkam et al. 2016).

A list of promising antioxidant bioactive peptides and their parent protein and algal sources are shown in Table 5. This free radical scavenging ability is recognised, although the structure-function relationship is poorly understood in comparison to carotenoids (Fan et al. 2014).

Table 5: Algae-derived antioxidant bioactive peptides (Fan et al. 2014)

peptide name or sequence	source	enzyme
protease extracts	*Scytosiphon lomentaria*	multienzyme complex
enzymatic digests	*Ishige okamurae*	multienzyme complex
VECYGPNRPQF	*Chlorella vulgaris*	
enzymatic hydrolysates	*Navicula incerta*	alcalase, Pronase-E, α-chymotrypsin, Neutrase, papain, pepsin, and trypsin
PGWNQWFL, VEVLPPAEL	*Navicula incerta*	alcalase, α-chymotrypsin, Neutrase, papain, pepsin, Pronase-E, and trypsin
LNGDVW	*Chlorella ellipsoidea*	papain, trypsin, pepsin, and α-chymotrypsin

peptide name or sequence	mechanism of action
protease extracts	hydroxyl, alkyl and DPPH radical
enzymatic digests	hydrogen peroxide
VECYGPNRPQF	hydroxyl radical, superoxide radical, peroxyl radical, DPPH radical and ABTS radicals
enzymatic hydrolysates	hydroxyl, superoxide, DPPH
PGWNQWFL, VEVLPPAEL	free radicals/antihepatotoxicity
LNGDVW	peroxyl, DPPH and hydroxyl radicals

Characterisation of antioxidant peptides from other food sources has shed light on common structural features, and this is important to guide future discovery of algal peptides. Meat proteins with antioxidant peptides are reported to commonly feature 4 – 16 amino acids and a molecular weight between 400 – 2500 Da, with the lower range of molecular weights generally possessing a higher antioxidant effect (Liu, R et al. 2016). A

shorter amino acid chain length, and therefore lower molecular weight, is also beneficial for passing through the intestinal barrier, and consequently increased bioavailability of the peptide (Roberts et al. 1999).

The presence of hydrophobic amino acids, such as leucine (L) and valine (V), in the N-terminal region and an overall hydrophobic nature of the peptide allows scavenging of free radicals from the lipid phase in the cell membrane (Liu, R et al. 2016). This was recognised in meat protein peptides but is also evident in the algae-derived antioxidant peptides shown in Table 5. Other common features recognised in antioxidant peptides from food protein sources include the presence of aromatic amino acids (F, W, Y) and sulphur-containing amino acids, such as cysteine (C) and methionine (M) (Nwachukwu & Aluko 2019).

1.3 Algae Health Benefits

Understanding the chemical structures and related biological effects highlighted in the 1.2 Chemistry of algae components section suggests the potential of these bioactive components in treatment or prevention of disease. Dysregulated metabolism, oxidative stress and inflammation are common features of many chronic diseases such as type 2 diabetes mellitus, cardiovascular disease, obesity, cancer and non-alcoholic fatty liver disease (NAFLD) (Biswas 2016; Hussain et al. 2016). Mitigating this dysregulation is important for maintaining health through later years of life as well, as ageing is also associated with these features (Biswas 2016). The prevalence of these underlying problems across a wide spectrum of disease states encourages the use of these algal compounds in treatment and prevention of multiple diseases. An overview of these potential health benefits will be explored in this section.

The antioxidant potency of the major algal carotenoids β-carotene, astaxanthin, fucoxanthin and lutein has been shown in numerous *in vitro* studies as discussed previously (Sachindra et al. 2007; Stahl & Sies 2003). These carotenoids have been tested in many animal and clinical studies to determine whether this antioxidant effect translates to meaningful health benefits. β-carotene has been extensively studied for its anti-cancer

potential, both in epidemiological and intervention studies in humans, but these studies have produced conflicting results (Fiedor & Burda 2014). Increased serum β-carotene concentrations are associated with reduced risk of mortality from cardiovascular disease, cancer, respiratory disease and diabetes mellitus over a 30-year period (Huang et al. 2018). However, when used to prevent cancer as an intervention in humans, β-carotene has usually produced no effects and in some cases, even adverse effects (Fiedor & Burda 2014; Group 1994; Lin et al. 2009). The reason for this lack of translation from epidemiological to intervention studies is not known but could be related to the isolation of β-carotene from other bioactive compounds in fruits and vegetables being detrimental (Rimm Eric 2018).

Astaxanthin has reported up to 10 times greater antioxidant activity than both β-carotene and lutein, and although there has not yet been testing in clinical trials for this purpose, there is the potential for a greater anti-cancer effect of this carotenoid. The astaxanthin clinical studies currently in progress are focusing on the antioxidant and anti-inflammatory changes in obesity, metabolic syndrome and osteoarthritis (ClinicalTrials.gov 2010, 2019a, 2019b). On the other hand, fucoxanthin has demonstrated promising anti-obesity effects in animal studies through reducing fat mass, fat deposition in the liver (risk factor for NAFLD), and fasting blood glucose and insulin concentrations (Koo et al. 2019; Maeda, Hosokawa, Sashima & Miyashita 2007; Sun et al. 2020b).

Sulphated polysaccharides are not broken down by human gastric enzymatic digestion and are also soluble in water, classifying them as soluble fibre. Soluble fibre attracts and binds water and, depending on the structure, can subsequently form a viscous gel during digestion (Chutkan et al. 2012). The formation of this viscous gel is considered to be beneficial as it increases satiety, lowers energy intake and improves blood lipid concentrations and glycaemic responses (Chutkan et al. 2012; Wanders et al. 2011). These effects are highly suited for prevention of obesity, type 2 diabetes and cardiovascular diseases such as atherosclerosis. Soluble fibre is also recognised as having activity as prebiotics, which are defined as fermentable compounds that confer beneficial changes to the composition and/or activity of the gut microbiome (Holscher 2017). Due to the

differences in structure between the sulphated polysaccharides, there are functions unique to each polysaccharide. Fucoidans are similar in structure to heparin, and consequently has been shown to exhibit anticoagulation and antithrombotic activities in a rat peritoneal inflammation model (Cumashi et al. 2007). This effect was not successfully translated to clinical studies, potentially due to low oral bioavailability (Irhimeh, Fitton & Lowenthal 2009). Sulphate groups interfere with the viral activity of enveloped viruses, such as human immunodeficiency virus and herpes simplex virus (Damonte, Matulewicz & Cerezo 2004). Carrageenans in particular have demonstrated an anti-viral effect, in both *in vitro* studies and one clinical trial (Leibbrandt et al. 2010; Ludwig et al. 2013). In this clinical trial, carrageenan was compared to a placebo spray and it was found that the duration of cold symptoms and viral load was reduced by the carrageenan nasal spray (Ludwig et al. 2013).

Polyphenols encompass an enormous group of compounds that have varied structures and health benefits. However, a common activity of these bioactive compounds is their well-described antioxidant and anti-inflammatory effects (Zhang & Tsao 2016). These biological effects are attributed to the polyphenol-induced reductions in blood pressure and plasma lipid concentrations, consequently lowering the risk of cardiovascular disease, and the improvement of insulin sensitivity to prevent type 2 diabetes (de Brito Alves et al. 2016; Gothai et al. 2016). The problem facing use of polyphenols for these health applications is poor bioavailability of these compounds and conflicting results from *in vivo* studies (de Brito Alves et al. 2016; Zhang & Tsao 2016). To date, there have been very limited clinical studies investigating the health benefits of phlorotannins. The first clinical trial in an obese population reported no improvement to biomarkers of inflammation and oxidative stress in overweight and obese participants (Baldrick et al. 2018). There was however a reduction in lymphocyte DNA damage, perhaps due to the antigenotoxic effect reported previously for eckol (Baldrick et al. 2018). Phlorotannins have effectively inhibited carbohydrate-digestive enzymes and reduced glucose-induced oxidative stress in animal studies, and this could have potential anti-diabetic effects so there is need for further testing in humans (Lee & Jeon 2013).

The anti-hypertensive and antioxidant effects of specific peptides were introduced earlier in the 1.2.4 Peptides section. This anti-hypertensive effect has been attributed primarily to the inhibition of the enzyme ACE, which is responsible for the conversion of angiotensin I to angiotensin II and the breakdown of bradykinin (Admassu et al. 2018). Consequently, inhibition of ACE leads to a decrease in blood volume and vasodilatation and as a result blood pressure is lowered (Turner & Kodali 2020)

Whilst effective ACE inhibitors, such as captopril, are already available on the market, there is still the potential for improved sustainability and efficacy and reduction of adverse effects (Herman et al. 2020). Natural products are generally sustainable, structurally diverse and have the potential for novel mechanisms of action that could aid in the development of new and improved ACE inhibitors or present a sustainable alternative (Yuan et al. 2016). Therefore, peptides derived from algae are worth further investigation and clinical trials are necessary to test these anti-hypertensive effects. Similarly, the antioxidant activities of algae-derived peptides have only been shown in *in vitro* studies and further testing in firstly animal models and then in clinical studies is required (Fan et al. 2014). It is evident that carotenoids, sulphated polysaccharides, polyphenols and peptides have the potential to treat or prevent a wide range of health conditions. Going forward, this literature review will focus on the use of these bioactive compounds in the prevention and treatment of metabolic syndrome.

1.4 Metabolic Syndrome

The term ‘metabolic syndrome’ is used to describe the co-occurrence of multiple cardiometabolic factors including central obesity, hypertension, insulin resistance, chronic low-grade inflammation and atherogenic dyslipidaemia more often in the same patient than would be predicted from the individual incidence in the community (Alberti et al. 2009). There are several different guidelines for clinical diagnosis of metabolic syndrome (Table 6).

The latest consensus panel in 2009 is the most commonly accepted guideline within Australia (Harris 2013). This stipulates that a patient must have at least three of the

following criteria: elevated waist circumference (central obesity), raised fasting plasma glucose concentrations, increased plasma concentrations of triglycerides, reduced high-density lipoprotein-cholesterol, or hypertension. Having these criteria increases the risk of developing cardiovascular disease (relative risk ~2.4) (Shin et al. 2013), type 2 diabetes (relative risk ~4.4) (Shin et al. 2013), certain cancers (e.g. pancreatic cancer in women relative risk 1.6) (Esposito et al. 2012) and overall all-cause mortality so it is important to recognise and treat these patients. However, it is apparent that current interventions are unable to stop the growing obesity and metabolic syndrome epidemic (O'Neill & O'Driscoll 2015).

Table 6: Definitions of metabolic syndrome

	NCEP ATP III (2001)	AHA/NHLBI (2004)	IDF (2005)	Consensus panel# (2009)
Criteria	Any three of the five criteria below	Any three of the five criteria below	Central obesity, plus two of the four criteria below	Any three of the five criteria below
Waist circumference	Male; >102 cm Female; >88 cm	Male; >102 cm Female; >88 cm	Population and country-specific definitions	Population and country-specific definitions
Fasting glucose	≥100 mg/dL	≥100 mg/dL	≥100 mg/dL or Rx	≥100 mg/dL or Rx
Dyslipidaemia (triglycerides)	≥150 mg/dL (1.7 mmol/L) or Rx*	≥150 mg/dL (1.7 mmol/L) or Rx*	≥150 mg/dL (1.7 mmol/L) or Rx*	≥150 mg/dL (1.7 mmol/L) or Rx*
Dyslipidaemia (HDL-cholesterol)	<40 mg/dL in males; <50 mg/dL in females or Rx*	<40 mg/dL in males; <50 mg/dL in females or Rx*	<40 mg/dL in males; <50 mg/dL in females or Rx*	<40 mg/dL in males; <50 mg/dL in females or Rx*
Hypertension	≥130/85 mmHg or Rx*	≥130/85 mmHg or Rx*	≥130/85 mmHg or Rx*	≥130/85 mmHg or Rx*

Rx stands for pharmacological treatment*
#Consensus panel includes World Heart Association, International Association for the Study of Obesity, IDF, NHBLI, and AHA
Abbreviations used: NCEP ATP III, National Cholesterol Education Program Adult Treatment Panel III; AHA, American Heart Association; NHLBI, National Heart, Lung, and Blood Institute; IDF, International Diabetes Foundation; HDL-c, high-density lipoprotein cholesterol.

It is estimated that one quarter of the world's adult population has metabolic syndrome and rates of obesity have doubled in 70 countries between 1980 and 2015 (Collaborators 2017; O'Neill & O'Driscoll 2015; Saklayen 2018). When considering that chronic disease accounted for 90% of all deaths in Australia in 2011, with coronary heart disease as the leading source, this increase is a major cause for concern (AIHW 2014). Understandably, this disease burden places huge demands on healthcare and reduces quality of life for those affected. Almost half of all Australians were estimated to have a chronic condition, and 20% are estimated to have two or more chronic conditions (Health & Welfare 2020). People aged 18-64 with two or more chronic conditions are 15% less likely to be working or seeking work and approximately 30% more likely to be experiencing psychological distress (Health & Welfare 2020).

The Australian government reported that costs of healthcare have increased in real terms (adjusted for inflation) by 5.4% per year from the period of 2001 to 2012, with cardiovascular disease topping the charts and accounting for 10% of all disease expenditure (AIHW 2014). The increase in the ageing population combined with the increase in disease risk factors, namely excess nutrition and inadequate physical activity, will only have led to increased expenditure and chronic disease prevalence in recent years (AIHW 2014). An effective method to deal with this growing problem is desperately needed.

It is necessary to understand the causes and modifiable factors of metabolic syndrome to be able to reduce the growing prevalence of the disease. The large-scale adoption of diets with increased saturated fats and simple carbohydrates combined with a sedentary lifestyle is considered responsible for the increase in incidence of metabolic syndrome (McGill 2014). This relationship between fat and carbohydrate intakes and risk of cardiovascular disease was examined in a large clinical study over a period of 24 – 30 years (Li et al. 2015). From this study, it can be observed in Figure 11 that saturated fats, *trans* fats and carbohydrates from refined or added sugar were all associated with an increased risk of cardiovascular disease, and their replacement with wholegrains or polyunsaturated fat alternatives decreased this risk (Li et al. 2015).

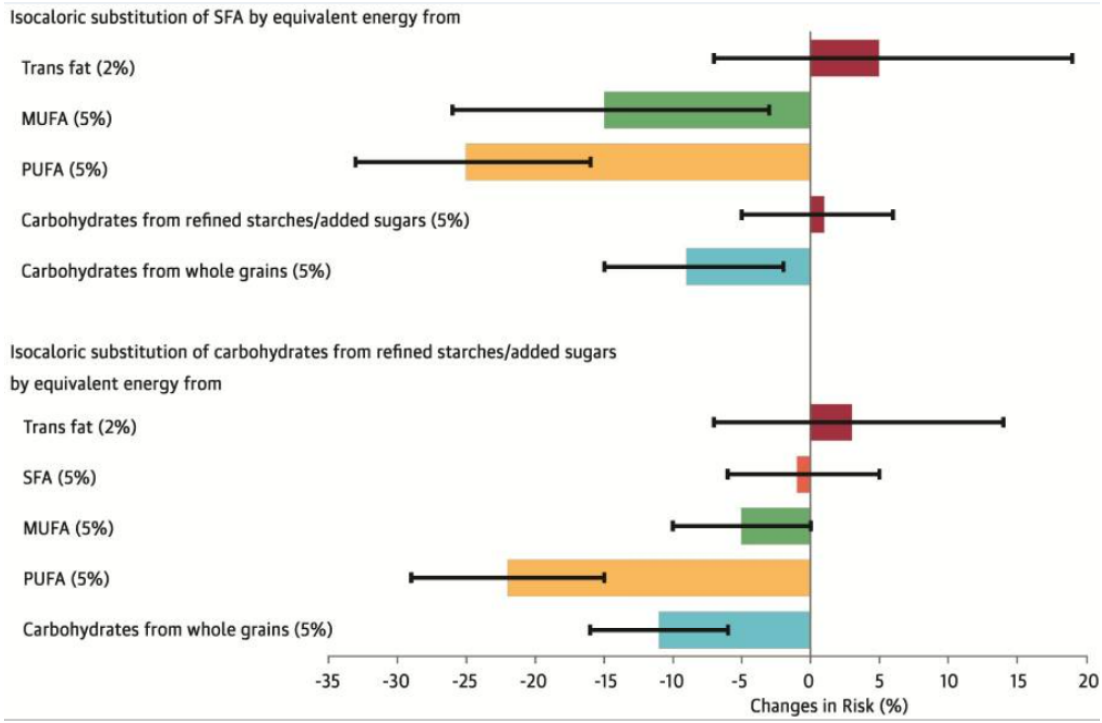


Figure 11: Estimated percent changes in the risk of coronary heart disease associated with isocaloric substitutions of one dietary component for another (Li et al. 2015)

There has been a similar association observed between consumption of highly processed foods such as sugar-sweetened beverages, pre-prepared frozen meals, ice cream, lollies and cookies with overweight and obesity prevalence in many countries (Poti, Braga & Qin 2017). The societal change in recent decades towards the reduced need for physical activity due to growth of technological entertainment and labour-saving options as well as the increased availability and relatively low cost of highly processed dietary options are believed to be a major contributor to this adoption of poor diet and sedentary lifestyle (Meldrum, Morris & Gambone 2017).

Genetic factors are also recognised to increase obesity risk. A well-known genetic causative factor is disruption of the leptin-melanocortin pathway due to genetic mutations, that consequently interferes with the normal function of leptin (Wasim et al. 2016). Leptin reduces food intake and increases energy expenditure, for example through regulating hepatic gluconeogenesis and lipolysis (Sáinz et al. 2015). In a cohort of 1013 morbidly

obese patients undergoing bariatric surgery, 3% were diagnosed with mutations to genes involved in the leptin-melanocortin pathway (Cooiman et al. 2020). This was supported by another study of 1230 obese adults and children, who reported that 3.9% of the total cohort were diagnosed with genetic markers of obesity and this percentage increased to 7.3% in the paediatric group (Kleinendorst et al. 2018). This proportion of patients was diagnosed with genetic obesity based on the current known genetic mutations, and there may be other genetic markers or biomarkers that are undiscovered and equally contribute to the risk of developing metabolic syndrome.

Whilst genetics and other metabolic factors are acknowledged to impact risk of metabolic syndrome, the rapid increase of metabolic syndrome prevalence suggests that these are minor factors in comparison to the major shift in lifestyle in recent decades (Saklayen 2018). It is possible that this increasingly obesogenic environment may be affecting certain genetic makeups more than others, which is supported by an increased association between genetic risk score and body mass index (BMI) at higher intakes of sugar-sweetened beverages (Qi, Q et al. 2012). Further research is needed to recognise the at-risk groups and to determine whether their risk can be mitigated.

Obese, pre-diabetic or type 2 diabetic patients are considered to be the major populations at risk of developing metabolic syndrome, and thus should be the focus of prevention strategies (Orchard et al. 2005; Scott 2003). Type 2 diabetes is characterised by hyperinsulinaemia, impaired glucose tolerance and insulin resistance (Orchard et al. 2005). It was observed in a clinical trial that, in a population of patients with impaired glucose tolerance (n = 3234), 53% of participants had metabolic syndrome at baseline. Further, controlling glucose concentrations of the remaining participants through either lifestyle or medication (metformin) reduced future development of metabolic syndrome (Orchard et al. 2005).

Obesity is a disorder characterised by chronic energy imbalance, whereby energy expenditure is consistently lower than energy intake, leading to the expansion of adipose tissue to maintain energy homeostasis. Healthy adipose tissue expansion requires increased recruitment of adipocyte precursor cells (hyperplasia) and appropriate increases in vascularisation and extracellular matrix growth (Sun, Kusminski & Scherer 2011).

Pathogenic expansion of adipose tissue is characterised by rapid adipose tissue growth necessitating adipocyte hypertrophy and increasing multiple cytotoxic stressors including endoplasmic reticulum stress, hypoxia and reactive oxygen species (Cinti et al. 2005; Sun, Kusminski & Scherer 2011). This difference between healthy adipose tissue and pathogenic adipose tissue is shown in Figure 12.

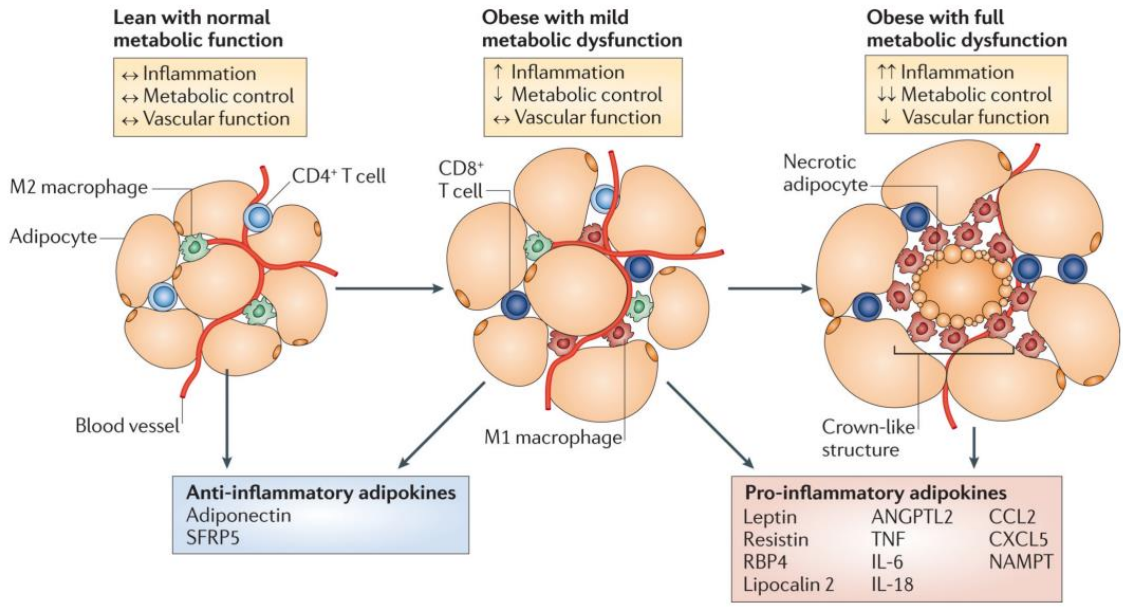


Figure 12: Adipose tissue changes in obesity and metabolic dysfunction (Ouchi et al. 2011)

An early stage in the progression towards metabolic dysfunction is the upregulation of $CD8^+$ T cells within adipose tissue, which promotes the recruitment and activation of the pro-inflammatory macrophages known as M1 macrophages (Wang & Wu 2018). This leads to the development of an inflammatory state within the adipose tissue, and together with adipocyte hypertrophy, excessive synthesis of the extracellular matrix and impaired capillary formation contributes to the death of adipocytes (Cinti et al. 2005; Lawler et al. 2016; Ouchi et al. 2011).

Adipocyte death is considered responsible for further recruitment and activation of M1 macrophages that phagocytose the lipid constituents released from the ruptured cell membrane and also secrete tumour necrosis factor-α (TNF- α), interleukin-1 (IL-1),

interleukin-6 (IL-6) and monocyte chemotactic protein-1 (Cinti et al. 2005; Lee, Wu & Fried 2010). These cytokines cause insulin resistance in adipocytes and also contribute to adipocyte dysfunction and loss of metabolic control (Xu et al. 2003). The accumulation of these macrophages around dead adipocytes is known as a crown-like structure and is a histological marker of obesity with metabolic dysfunction (Ouchi et al. 2011). This excess adiposity and adipocyte dysfunction deregulates adipokine secretion (Jung & Choi 2014).

Adipose tissue secretes many adipokines including IL-6, TNF-α, leptin, adiponectin and angiotensinogen (Hajer, van Haeften & Visseren 2008). As stated earlier, IL-6 and TNF-α further contribute to the state of insulin resistance and chronic inflammation characteristic of obesity with metabolic dysfunction (Kern et al. 2001). Leptin reduces food intake and increases energy expenditure, for example through regulating hepatic gluconeogenesis and lipolysis (Sáinz et al. 2015). Leptin concentrations are chronically elevated in obesity suggesting resistance to leptin, and this resistance is correlated with elevated cytokines (e.g. TNF-α), insulin resistance and elevated triglycerides (Leon-Cabrera et al. 2013).

On the other hand, adiponectin synthesis is reduced in obesity, insulin resistance and type 2 diabetes (Hajer, van Haeften & Visseren 2008). Adiponectin is responsible for improving insulin sensitivity, increasing fatty acid oxidation in both liver and muscle tissue and enhancing uptake of glucose by muscles (Hajer, van Haeften & Visseren 2008). Angiotensinogen is the precursor of angiotensin II, which increases blood pressure by constricting blood vessels. Angiotensinogen expression in visceral adipose tissue is increased in central obesity and systolic blood pressure was not elevated in obese mice when this gene was knocked out suggesting the important role of adipose angiotensinogen in obese hypertension (Rahmouni et al. 2004; Yiannikouris et al. 2012).

Obesity is also linked to increased prevalence of dyslipidaemia, characterised by elevated fasting and postprandial triglycerides, increased small, dense low-density lipoprotein (LDL) and reduced high density lipoprotein (HDL) cholesterol (Klop, Elte & Cabezas 2013). Dyslipidaemia is considered a major risk factor for developing atherosclerosis, and consequently increasing the risk of myocardial infarction and stroke (Klop, Elte & Cabezas 2013). It is evident that central obesity is associated with even higher risk of

insulin resistance, oxidative stress, hypertension, chronic-low grade inflammation and atherogenic dyslipidaemia (Tchernof & Després 2013; Wing et al. 2011). The similarity of this risk profile with metabolic syndrome criteria prompted the IDF to declare central obesity an essential component of metabolic syndrome in 2005 (Table 6). This has since been changed to reflect variation as some lean patients develop metabolic syndrome; however, central obesity is still recognised as a major factor in metabolic syndrome development and disease progression.

Metabolic syndrome increases the risk of developing chronic diseases, which reduces quality of life and can be fatal. Insulin resistance and increased serum free fatty acids (FFA) lead to non-alcoholic fatty liver disease (NAFLD), whereby fat accumulates in the liver as triglycerides (Buzzetti, Pinzani & Tsochatzis 2016). This fatty build-up can trigger chronic inflammation within the liver, known as steatohepatitis, which damages the liver and can progress to fibrosis and even cirrhosis (Buzzetti, Pinzani & Tsochatzis 2016). The only treatment for this advanced stage of cirrhosis is liver transplantation.

Osteoarthritis is another incurable complication of metabolic syndrome, independent of sex or race, but this association is diminished in populations over 65 years old (Puenpatom & Victor 2009). In particular, metabolic syndrome symptoms of hypertension, obesity and hyperglycaemia were identified as being linked to osteoarthritis possibly through the increased concentration of leptin (Puenpatom & Victor 2009; Sekar et al. 2017; Sellam & Berenbaum 2013). Metabolic syndrome has also been linked to an increased risk of developing cancer, in particular liver, colorectal, rectal, pancreatic and sex-specific cancers such as endometrial and breast in postmenopausal women (Esposito et al. 2012).

Mechanisms linking metabolic syndrome to these conditions are not fully understood, but it is suggested that chronic low-grade inflammation, glucose and lipid toxicity and adipokine dysregulation may be responsible (Esposito et al. 2012; Sellam & Berenbaum 2013). It is now becoming apparent that another major factor in the development of metabolic syndrome and obesity is the gut microbiome (Tilg & Kaser 2011).

1.5 Gut Microbiome

The human gastrointestinal tract is colonised by up to 10 trillion microbes, found predominantly within the colon (Sender, Fuchs & Milo 2016). These microbes are collectively referred to as the gut microbiome, of which 99% comprises bacteria and the remainder includes fungi, viruses and protozoa (Qin et al. 2010). However, it is believed that the proportion of bacteriophages (virus) are being greatly underestimated due to their lack of conserved regions and embedment in bacterial chromosomes making classification difficult (Weiss & Hennet 2017). Only limited fungal species are able to colonise the gut, and their prevalence is heavily controlled through fungi colonisation of the food consumed by the host (Hallen-Adams & Suhr 2016). The prevalence of certain species of fungi and bacteria are correlated, suggesting that there is a symbiotic relationship (Hoffmann et al. 2013).

Despite the large number of bacteria, there is limited phylum diversity within the gut microbiome community, as Bacteroidetes and Firmicutes account for 90% of total bacteria (Rinninella et al. 2019). The common taxonomic composition is shown in Figure 13, along with the other phyla that form the gut microbiome in smaller proportions including Actinobacteria, Proteobacteria, Fusobacteria and Verrucomicrobia. The average person carriers ~160 different species, although this can range up to 400, and there are at least 1000 different gut bacterial species that have been characterised (Lloyd-Price, Abu-Ali & Huttenhower 2016). There is even further variation within these species based on their strain, which can possess major genetic differences (Greenblum, Carr & Borenstein 2015). It is understood that the first three years of life are critical in the development of an individual's unique gut microbiome, particularly the history of breastfeeding and mode of delivery at birth (Xu & Knight 2015). Subtle changes still occur after this rapid early colonisation, and this is in response to dietary habits, body mass index (BMI), exercise frequency and other environmental factors such as geographical location (Rinninella et al. 2019).

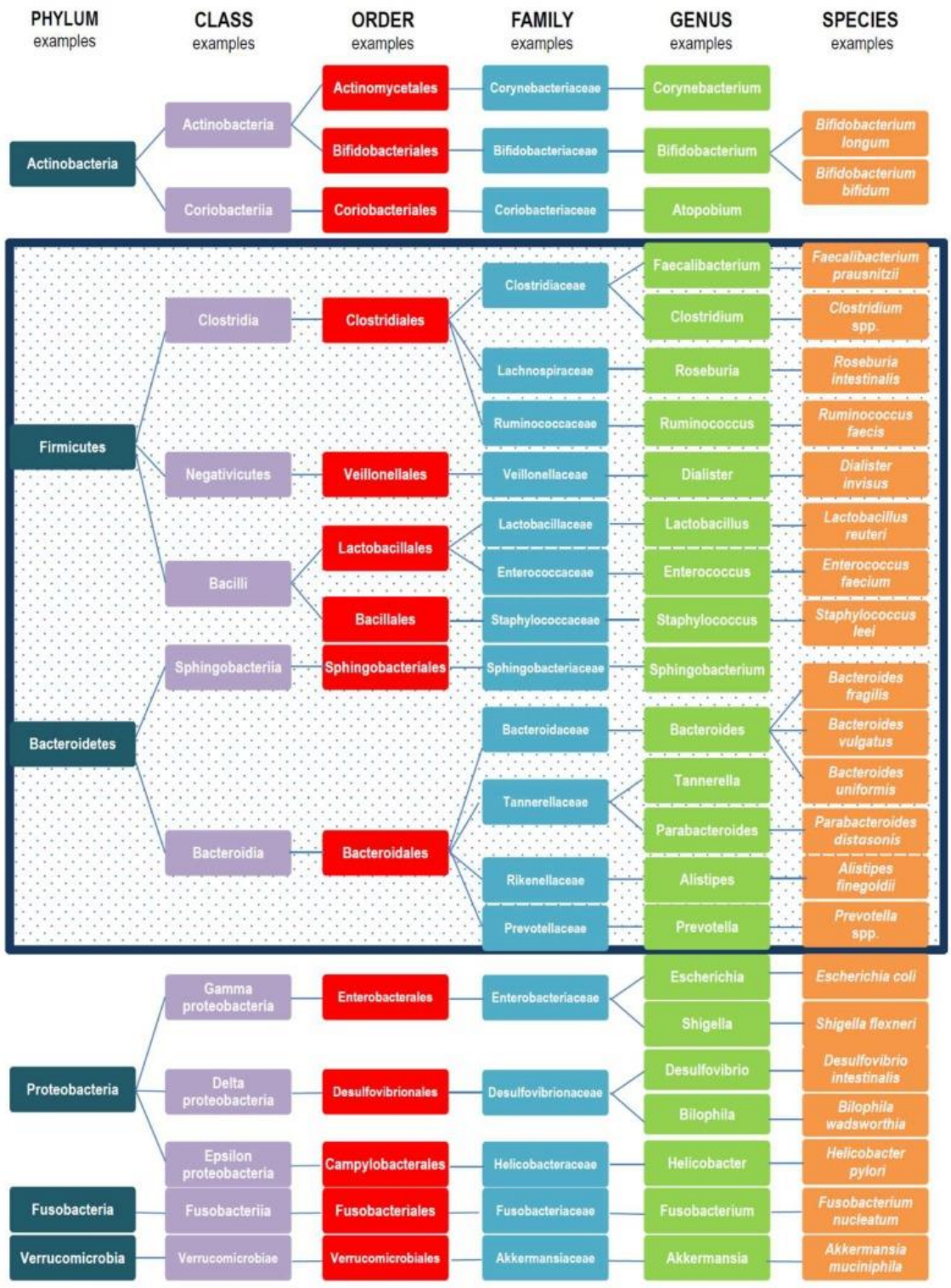


Figure 13: Taxonomic gut bacterial microbiota composition (Rinninella et al. 2019)

The diversity and prevalence of certain species can indicate whether a gut microbiome is 'healthy' or implicated in disease (Kolde et al. 2018). A healthy gut microbiome is ecologically diverse, meaning that the species variety is rich with even abundance amongst different microbes (Lloyd-Price, Abu-Ali & Huttenhower 2016). This diversity promotes functional variety, enabling the gut microbiome to effectively maintain the integrity of the mucosal barrier lining the gastrointestinal tract, utilise non-digestible dietary carbohydrates to synthesise short-chain fatty acids (SCFA), prevent pathogen colonisation and produce vitamins for the host (Kovatcheva-Datchary & Arora 2013).

Another important role is the beneficial interaction between the gut microbiota and the mucosal immune system, mediated through SCFA absorption and interaction with the innate immune system (Kau et al. 2011). On the other hand, dysregulation of the gut microbiome caused by either loss of beneficial organisms, irregular bacteria blooms or a loss in overall diversity is known as dysbiosis and is implicated in disease (Dabke, Hendrick & Devkota 2019). A common feature of dysbiosis is an increased ratio of Firmicutes to Bacteroidetes (Koliada et al. 2017). The factors leading to development of dysbiosis are not completely understood, but genetic factors and a diet high in saturated fats and simple carbohydrates but low in dietary fibre are believed to have a causative role (DeGruttola et al. 2016). Dysbiosis is associated with many disease states such as inflammatory bowel disease, obesity, diabetes and cardiovascular disease (Dabke, Hendrick & Devkota 2019; DeGruttola et al. 2016).

The mechanisms linking dysbiosis to development of metabolic disease are shown in Figure 14 (Dabke, Hendrick & Devkota 2019). Dysbiosis alters the metabolites produced by the gut microbiome, resulting in the loss of glucagon-like peptide-2 (GLP-2) and the upregulation of lipopolysaccharides (LPS) and other aberrant metabolite release (Dabke, Hendrick & Devkota 2019). GLP-2 helps maintain tight junctions between intestinal epithelial cells, which is essential for regulating the movement of substances from the lumen of the intestine into the bloodstream (Cani et al. 2009).

Loss of the protective GLP-2 allows endotoxins such as LPS to pass into the bloodstream raising plasma concentrations (Dabke, Hendrick & Devkota 2019). LPS are a major component of the outer membrane of gram-negative bacteria and have been confirmed to

contribute to the chronic inflammation of the liver and adipose tissue increasing the risk of NAFLD and adipose tissue dysfunction (Cani et al. 2009; DeGruttola et al. 2016). The *Enterobacteriaceae* family within the proteobacteria phylum (Figure 13) are an example of a pathogenic gram-negative bacteria that release LPS leading to inflammation (Weiss & Hennet 2017). Bacteriophages perhaps contribute to this release of LPS through the lysis of the bacterial host and subsequent release of intracellular toxins, cell wall fragments and lipids (Weiss & Hennet 2017).

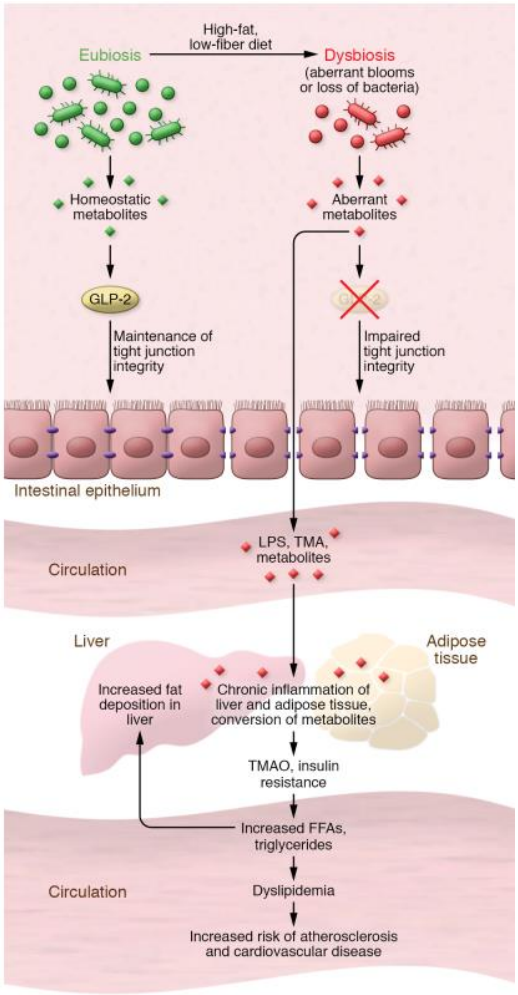


Figure 14: Gut microbiome interaction with the intestinal barrier and contribution to metabolic disease (Dabke, Hendrick & Devkota 2019)

Prebiotics, commonly soluble fibre as discussed earlier, are used to attempt to prevent or reverse dysbiosis (Weiss & Hennet 2017). Prebiotics can selectively increase the growth

of specific bacteria through providing a substrate for fermentation, and consequently SCFA are produced, and antibacterial secretion and anti-adhesive degradative products prevent pathogen growth (Pourabedin & Zhao 2015). Probiotics can potentially provide a similar benefit, as they contain live microorganisms chosen specifically for their functional characteristics with the purpose of populating the gut with beneficial bacteria (Bermudez-Brito et al. 2012). Due to the importance of the gut microbiome in multiple disease states, as discussed earlier, preventing dysbiosis and promoting growth of beneficial bacteria through use of prebiotics and probiotics is a possible treatment option. For example, it has been observed that NAFLD patients have lower abundance of both *Alistipes* and *Prevotella* than healthy subjects, and this may be a potential treatment or prevention option (Jiang et al. 2015).

1.6 Current Metabolic Syndrome Treatments

Due to the cardiometabolic factors that coexist in metabolic syndrome, patients often experience multiple health conditions that require individual treatments. Type 2 diabetes is often treated with metformin to improve insulin sensitivity and control glucose concentrations. The increased risk of developing hypertension and dyslipidaemia have been discussed earlier, and both conditions require pharmaceutical treatment such as ACE inhibitors or statins, respectively. There are no pharmaceutical treatment options for NAFLD and, in end-stage disease, the only option is liver transplantation (Romero-Gómez, Zelber-Sagi & Trenell 2017). The underlying chronic low-grade inflammation and oxidative stress implicated in development and progression of these chronic diseases is also not targeted by current pharmaceutical options (Minihane et al. 2015).

Obesity with the underlying lifestyle problems of physical inactivity and excess intake of foods with increased saturated fats and simple carbohydrates has proven to be more problematic to overcome as prevalence of obesity is increasing (McGill 2014). Changing lifestyle is difficult for patients to implement and even harder to maintain (Martin et al. 2005). For example, lifestyle advice including exercise has only a 19-35% patient adherence rate (Martin et al. 2005). This high attrition rate is increased in patients who

have attempted dieting before, or who have unrealistic expectations for weight loss (Moroshko, Brennan & O'Brien 2011). Of even more concern is that, after successfully completing a lifestyle intervention program, patients on average regain one third of their lost weight in the following year and reach their baseline weight in four to five years (Webb & Wadden 2017). Consequently, bariatric surgery and prescription weight loss drugs are used to increase the likelihood of inducing and maintaining weight loss.

Bariatric surgery increased average weight loss of patients by 26 kg more than patients who used non-surgical treatments, with an average weight loss of 30 kg and 4 kg respectively (Gloy et al. 2013). Another study that followed participants for 10 years after bariatric surgery and compared them to nonsurgical matches found that this substantial weight loss that occurs within the first year is able to be maintained long-term (Figure 15) (Maciejewski et al. 2016). This is in direct contrast to the relapsing nature of obesity after lifestyle changes as discussed above, as only 3.4% of patients who underwent bariatric surgery regained weight back to within 5% of their baseline weight (Maciejewski et al. 2016).

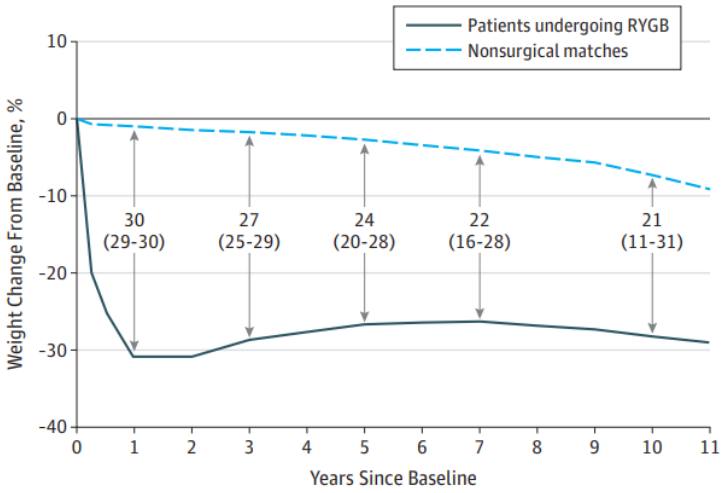


Figure 15: Differences in estimated weight changes among patients undergoing Roux-en-Y gastric bypass (RYGP) and nonsurgical matches (Maciejewski et al. 2016)

The most effective and commonly performed bariatric surgery method is Roux-en-Y gastric bypass (RYGP) (Maciejewski et al. 2016). RYGP involves the attachment of the

jejunum to the upper part of the stomach, and consequently most of the stomach and the duodenum are bypassed (Figure 16) (Lupoli et al. 2017). The duodenum is the first section of the small intestine and is the major site for digestion and absorption of nutrients (Lupoli et al. 2017). Bypassing most of the stomach and duodenum reduces food capacity and energy intake from the food consumed due to malabsorption (English & Williams 2018). Adversely, this malabsorption can result in vitamin and mineral deficiency leading to anaemia and is associated with abnormalities in bone metabolism (Gagnon & Schafer 2018; Lupoli et al. 2017).

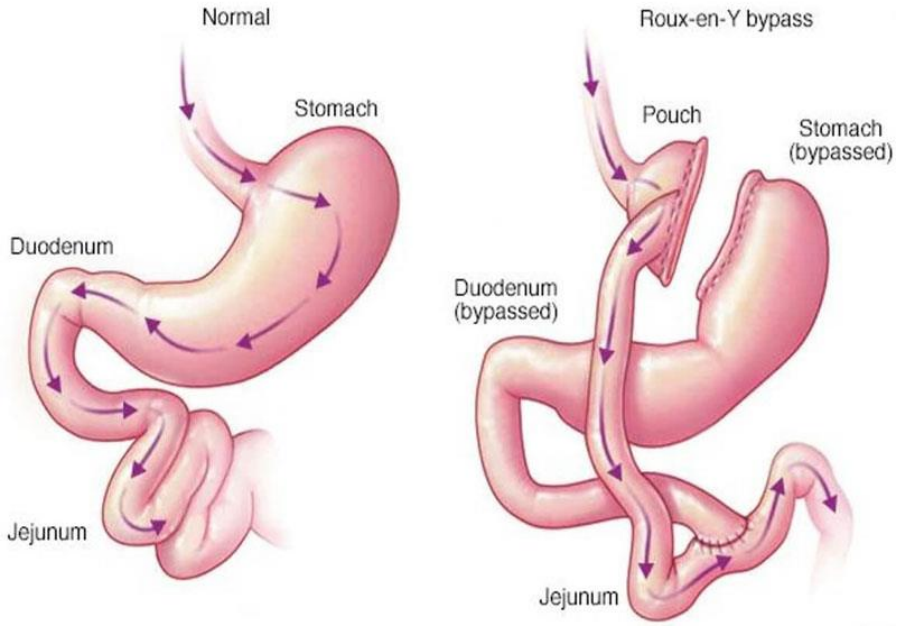


Figure 16: Roux-en-Y gastric bypass (Surgery 2020)

Bariatric surgery is also not universally available due to cost and there is the low risk of 30-day mortality following surgery (0.3%) as severely obese patients generally have higher risk factors for cardiovascular complications and the nature of the surgery being gastrointestinal has further risks of local infection or sepsis (Smith et al. 2011). These concerns and the irreversibility of the surgery may be why in the United States in 2018 only 1.1% of patients who qualified for bariatric surgery underwent the procedure (English et al. 2020; Hennings et al. 2018).

Prescription weight loss drugs currently available for long-term use are listed in Table 7 (Tak & Lee 2020). These drugs are recommended as an adjunct to lifestyle changes, rather than a replacement, and they produce an estimated 3 – 7% weight loss over one year depending on the drug (Srivastava & Apovian 2018). This was achieved primarily through suppression of appetite, although orlistat instead decreases lipid absorption, and due to this mechanism of action there are other unwanted side effects (Table 7) (Tak & Lee 2020). Concern surrounding these adverse side effects, as well as the withdrawal of two previously approved weight loss drugs from the market (sibutramine and lorcaserin) in the last 10 years due to increased risk of cardiac arrest and cancer, may have contributed to the low utilisation of weight loss drugs (Elangovan, Shah & Smith 2020). In fact, only 2.9% of all adults with obesity in the United States were prescribed weight loss medication in 2019 (Elangovan, Shah & Smith 2020). This has encouraged the pursuit of alternative methods such as functional foods that could replace weight loss drugs on the market to treat obesity without being hindered by toxicity or poor patient compliance.

Table 7: A summary of prescription weight loss drugs (Tak & Lee 2020)

Drug	Product name	Application	Mechanism of action	Main adverse effect	Contraindication	FDA approval	EMA approval	Korea approval
Orlistat	Xenical®, Alli®	60 or 120 mg TID during or within 1 hour of a fat-containing meal	Gastrointestinal and pancreatic lipase inhibitor; decrease lipid absorption	Oily stools, oily spotting, fecal urgency, fecal incontinence, hyperdefecation, flatus with discharge, deficiency in vitamins A, D, E, and K	Pregnancy, cholestasis, malabsorption	Yes 1999	Yes 2012	Yes 2000
Phentermine/ topiramate	Qsymia®	3.75/23 mg QD for 14 days and then 7.5/46 mg QD; If <3% weight loss is achieved at 12 weeks, increase to 11.25/69 mg QD for 14 days, followed by 15/92 mg QD; discontinue gradually if <5% weight loss is achieved at 12 weeks with the highest dose	NE agonist/GABA agonist, glutamate antagonist; suppress appetite	Paresthesia, dry mouth, constipation, insomnia, dysgeusia, anxiety, depression	Pregnancy, uncontrolled HTN, CVD, CKD, glaucoma, hyperthyroidism patients on MAOIs	Yes 2012	No	Yes 2019
Naltrexon/ bupropion	Contrave®, Mysimba®	8/90 mg for 7 days; BID for 7 days; 2 tablets in the morning and 1 tablet in the evening for 7 days; and 2 tablets BID thereafter	Opioid receptor antagonist/dopamine agonist and NE reuptake inhibitor; increase satiety, suppress appetite	Nausea, headache, constipation, dizziness, vomiting, dry mouth	Pregnancy, uncontrolled HTN, seizure, anorexia or bulimia nervosa, abrupt discontinuation of alcohol, benzodiazepines, barbiturates or antiepileptic drugs, other bupropion-containing drugs, opioids or opiate agonists, MAOIs	Yes 2014	Yes 2015	Yes 2016
Liraglutide	Saxenda®	0.6 mg subcutaneous injection QD, increase by 0.6 mg weekly to a daily target dose of 3 mg	Glucagon-like peptide-1 agonist; slow gastric emptying, increase satiety, decrease food reward	Nausea, diarrhea, constipation, vomiting, dyspepsia	Pregnancy, personal or family history of medullary thyroid carcinoma or type 2 MEN	Yes 2014	Yes 2015	Yes 2017
Lorcaserin	Belviq®, Belviq XR®	10 mg BID 20 mg extended release QD	Serotonin 2C receptor agonist; reduce food intake	Headache, dizziness, fatigue, nausea, constipation, dry mouth	Pregnancy, severe renal disease	Yes 2012 Withdrawn from the market in February 2020	No	Yes 2015 Withdrawn from the market in February 2020

1.7 Functional Foods

Functional foods are defined as foods that not only provide nutrition, but also have the capacity to treat or prevent disease (Brown, Poudyal & Panchal 2015). The interest in health benefits of foods that have been used for thousands of years as traditional medicines, such as algae, has been increasing in the last few decades as an alternative to pharmacological treatment (Plaza, Cifuentes & Ibáñez 2008). Functional foods have the capacity for a wide variety of health benefits without producing serious side effects, although to date there has not been substantial evidence to support their use in a clinical setting (Brown, Poudyal & Panchal 2015). A major benefit of functional foods is the capacity to not only target obesity, but simultaneously address the metabolic and cardiovascular problems prevalent in metabolic syndrome (Brown, Poudyal & Panchal 2015). The wide range of bioactive compounds found in algae and their reported health benefits as discussed in the 1.3 Algae Health Benefits section advocate strongly for the use of algae as functional foods.

Edible algae have been consumed for thousands of years, and remain a staple of the Japanese diet in particular to this day (Pérez-Lloréns et al. 2020). Commonly consumed brown algae species such as *Undaria pinnatifida (*wakame) and *Laminaria japonica* (kelp) have been shown to produce antioxidant and anti-diabetic effects *in vitro* (Liu, B et al. 2016). Further, a high dosage of 10% *U. pinnatifida* or *L. japonica* provided to rats for four weeks suppressed weight gain (Kim et al. 2016). This was suggested to be due to the increased prevalence of beneficial bacteria and decreased pathogenic bacteria in the gut microbiome, induced by the high dietary fibre content of these seaweeds, particularly *L. japonica* (50.7% total dietary fibre) (Kim et al. 2018).

Tropical and sub-tropical seaweeds such as *Porphyra* spp. and *Ulva* spp. are consumed in Vietnam, and commonly used as ingredients in soup or as an accompaniment to a meal (Hong, Hien & Son 2007). Globally, *Porphyra* spp. are consumed and recognised as nori, the seaweed used as the outer layer of sushi (Venkatraman & Mehta 2019). In addition to containing relatively high amounts of protein, carbohydrates and micronutrients, *Porphyra* contain bioactive peptides and sulphated polysaccharides that have induced

anti-hypertensive and antioxidant effects (Venkatraman & Mehta 2019). These are just several examples of the many potential functional foods derived from algae species (Mohamed, Hashim & Rahman 2012). However, there is concern whether the heavy metal content of algae may cause adverse health effects as algae possess multiple metal binding sites (Roleda et al. 2019). The heavy metal content fluctuates considerably due to variable environmental exposure to heavy metals, so, even though the average heavy metal content is within safe guidelines (Table 8), there is still cause for possible concern (Paz et al. 2019).

Table 8: Estimated daily intake (EDI) values and contribution percentages of the analyzed elements from the most consumed edible seaweeds in Europe. Assuming adult weight of 68.48 kg (Paz et al. 2019)

Element	Parameter	Guideline values	Wakame (*Undaria pinnatifida*)				Sea spaghetti (*Himanthalia elongata*)		Kombu (*Laminaria ochroleuca*)	
			Europe		Asia		Europe		Europe	
			EDI (mg/day)	Contribution (%)	EDI (mg/day)	Contribution (%)	EDI (mg/day)	Contribution (%)	EDI (mg/day)	Contribution (%)
Ca	UL	2000 mg/day	16.6	0.83	19.6	0.98	17.3	0.87	14.6	0.73
B		20 mg/day	0.35	1.75	0.13	0.65	0.16	0.80	0.19	0.95
Cu		10000 µg/day	0.009	0.09	0.01	0.10	0.01	0.10	0.01	0.10
Fe		45 mg/day	0.11	0.24	0.29	0.64	0.09	0.20	0.14	0.31
Mn		11 mg/day	0.01	0.13	0.03	0.27	0.07	0.64	0.04	0.36
Mo		2 mg/day	0.0006	0.03	0.0004	0.02	0.0004	0.02	0.0004	0.02
Zn		40 mg/day	0.20	0.50	0.09	0.23	0.11	0.28	0.05	0.13
V		1.8 mg/day	0.0005	0.03	–	–	0.009	0.50	0.003	0.17
Mg		< 250 mg/day	13.8	5.52	23.3	9.32	17.7	7.10	8.27	3.31
Cr	TDI	0.3 mg/kg bw/day	0.001	< 0.01	0.001	< 0.01	0.003	< 0.01	0.001	< 0.01
Ba		0.02 mg/kg bw/day	0.02	1.46	0.008	0.58	0.02	1.46	0.03	2.20
Ni		2.8 µg/kg bw/day	0.002	1.04	0.002	1.04	0.008	4.17	0.002	1.04
Na	ADI	≤ 2000 mg/day	98.9	4.95	141	7.05	125	6.25	75.2	3.76

Abbreviations used: UL, tolerable upper intake level; TDI, tolerable daily intake; ADI, acceptable daily intake; EDI, estimated daily intake.

Identifying the components providing the health benefits within algae can suggest future selection of algae functional foods and guide cultivation of algae to contain the optimal nutrient and bioactive composition. For example, it was discussed earlier that the carotenoid content in algae can be controlled through placing the algae under stress conditions. Testing the individual algae compounds will also guide selection of the optimal dose, which can further guide improvement of algae composition for use as functional foods. The unique algae compounds discussed previously will be the focus for the remainder of this review.

The carotenoids fucoxanthin (Table 9) and astaxanthin (Table 10) have been tested in models of obesity and metabolic syndrome in animals and in several clinical trials.

Table 9: Fucoxanthin previous studies

Animal Model/Human	Fucoxanthin Dose (Duration)	Effects on Metabolic Syndrome	Mechanisms
KK-Ay mice (Hosokawa et al. 2010) Obese, diabetic strain	0.2% diet (4 weeks)	↓ body weight ↓ white adipose tissue ↓ non-fasting blood glucose ↑ brown adipose tissue	↓ adipocytokines ↓ macrophage infiltration in white adipose tissue ↔ adiponectin
C57BL/6N mice (Woo et al. 2009) High-fat diet (20% fat)	0.05% and 0.2% diet (6 weeks)	0.05% and 0.2% performed equally ↓ body weight ↓ white adipose tissue ↓ feed efficiency ↔ brown adipose tissue	↓ leptin ↓ lipogenic enzymes ↑ β-oxidation ↑ adiponectin (0.05% dose performed best) ↑ UCP1-3 expression
KK-Ay mice (Maeda et al. 2015) Obese, diabetic strain	0.1% diet (27 days)	↔ body weight ↔ white adipose tissue ↔ brown adipose tissue ↓ blood glucose after glucose tolerance test	↓ adipocytokines
Wistar rats (Grasa-López et al. 2016) High-fat (20%), high-carbohydrate (48.5%) diet	1 mg/kg body weight (8 weeks)	↔ body weight ↓ intra-abdominal fat ↔ retroperitoneal fat ↓ triglycerides ↓ LDL/VLDL cholesterol ↑ HDL cholesterol ↓ blood glucose after insulin tolerance test ↓ systolic and diastolic blood pressure ↓ adipocyte size	↑ adiponectin ↓ leptin ↑ UCP1 expression ↑ PPARγ expression ↑ PGC1α expression
KK-Ay mice (Beppu et al. 2012) Obese, diabetic strain	0.2% diet (4 weeks)	↓ epididymal white adipose tissue ↑ HDL cholesterol ↑ non-HDL cholesterol ↑ liver weight ↓ liver cholesterol content	Cholesterol synthesis ↑ SREBP1 expression ↑ SREBP2 expression Cholesterol clearance ↓ liver LDL-receptor expression ↓ SR-B1 expression
Japanese adults with BMI > 25 (Hitoe & Shimoda 2017)	1 and 3 mg daily (4 weeks)	3 mg daily ↓ visceral fat area cm^2 1 mg daily ↓ total fat area cm^2 ↓ subcutaneous fat area cm^2	

Abbreviations used: UCP1, uncoupling protein 1; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; PPAR, peroxisome proliferator-activated receptor; PGC1, proliferator-activated receptor-gamma coactivator-1; SREBP2, sterol regulatory element-binding protein 2; SR-B1, scavenger receptor, class B type 1.

Table 10: Astaxanthin previous studies

Animal Model/Human	Astaxanthin Dose (Duration)	Effects on Metabolic Syndrome	Mechanisms
Albino mice (swiss strain) (Bhuvaneswari et al. 2010) High-fat (20%), high-fructose (45%) diet	6 mg/kg body weight/day (day 16 – day 60)	↓ body weight ↓ liver weight ↓ liver cholesterol, triglyceride and free fatty acids ↓ fasting blood glucose ↓ fasting insulin ↓ AST and ALT ↓ plasma triglyceride and free fatty acids	Antioxidant enzymes ↑ superoxide dismutase ↑ catalase ↑ glutathione peroxidase
C57BL/6J mice (Yang et al. 2014) High-fat (35%) diet	0.003, 0.01 or 0.03% diet (12 weeks)	0.03% diet ↔ body weight ↔ retroperitoneal fat ↔ liver weight, triglyceride, and cholesterol content ↓ plasma triglyceride ↔ total cholesterol ↔ HDL cholesterol ↓ AST	0.03% diet Antioxidant enzymes ↑ superoxide dismutase ↑ nuclear factor erythroid 2-related factor 2 ↑ glutathione peroxidase
C57BL/6J mice (Kim et al. 2017) High-fat (35%), high-sucrose (35%) diet	0.03% diet (30 weeks)	↔ body weight ↔ liver weight, triglyceride, and cholesterol content ↔ epididymal fat ↓ retroperitoneal fat ↔ total cholesterol ↔ triglycerides	↔ lipogenesis genes ↓ reduced collagen in epididymal fat ↓ expression of macrophage markers in epididymal fat
Mildly hyperlipidaemic adults (Yoshida et al. 2010)	6, 12, or 18 mg/day (12 weeks)	6, 12 and 18 mg daily ↓ serum triglyceride ↑ HDL cholesterol ↔ LDL cholesterol ↔ total cholesterol	12 and 18 mg daily ↑ adiponectin
Adults with type 2 diabetes (Mashhadi et al. 2018)	8 mg/day (8 weeks)	↓ visceral fat mass ↔ body mass intake ↔ total fat mass ↓ serum triglyceride ↓ VLDL cholesterol ↔ HDL cholesterol ↔ LDL cholesterol ↓ systolic blood pressure ↔ diastolic blood pressure ↓ fructosamine concentration ↔ fasting blood glucose	↑ adiponectin

Abbreviations used: HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; ALT, alanine transaminase; AST, aspartate aminotransferase.

The focus of previous fucoxanthin studies has been to test the potential anti-obesity effects in mice and rat studies and one clinical trial. The results thus far have been conflicting as body weight or fat mass were only reduced in 4 of 6 studies. Early investigation into mechanisms of action suggests that fucoxanthin is inducing this weight loss and other reductions in blood glucose and blood pressure through decreasing adipocytokines, lipogenic enzymes and upregulating UCP1 expression to increase thermogenesis (Grasa-López et al. 2016; Hosokawa et al. 2010; Woo et al. 2009). One study reported an increased concentration of LDL-cholesterol due to upregulated synthesis of cholesterol and decreased clearance, potentially increasing the risk of cardiovascular disease and should be further explored in future studies (Beppu et al. 2012). The lowest effective dose when fucoxanthin was provided in the diet was 0.05%, which performed equally to the 0.2% dose (Woo et al. 2009). The conflicting results surrounding the effect of fucoxanthin on cholesterol concentrations, obesity, and blood glucose management, suggest that further animal studies are recommended to determine whether there is merit in attempting another clinical trial. There has only been one study to date that has tested the effect of fucoxanthin in humans (Mashhadi et al. 2018), and they reported minimal success in translating the same health benefits that were observed in previous animal studies, suggesting that potentially the low doses used were not sufficient.

Astaxanthin reduced body weight in only one study in mice (Bhuvaneswari et al. 2010) thus far, however abdominal fat mass was reduced in an additional two studies (Kim et al. 2017) including one in humans (Mashhadi et al. 2018). Despite this, astaxanthin has been unable to reduce body weight in humans at a dose ranging from 6 – 20 mg/day tested in multiple clinical trials that were undertaken for up to 3 months (Xia et al. 2020). The most consistently reported health benefits include reduction of blood triglyceride concentrations, which was reported in 4 of 5 studies, and an antioxidant effect. In adults, early indications suggests that there appears to be no benefit to increasing the dose above approximately 6 mg/day (Choi et al. 2011; Yoshida et al. 2010). In rodents, the dose that produced the greatest health benefits was 6 mg/kg body weight/day (Bhuvaneswari et al. 2010). It is recognised that astaxanthin has poor bioavailability, and this may be why the anti-obesity effects have not yet been translated to humans (Mercke Odeberg et al. 2003). There was an increased anti-obesity effect of fucoxanthin when it was provided in the diet

with medium-chain triglycerides, due to the fat solubility of carotenoids (Maeda, Hosokawa, Sashima, Funayama, et al. 2007). Evidence of this effect with astaxanthin in an animal trial, may suggest that another clinical trial, this time combining astaxanthin with a lipid-based formulation, could result in an improved translation of health benefits to humans.

The sulphated polysaccharides ulvan (Table 11) and fucoidan (Table 12) have been tested in models of obesity and metabolic syndrome in animals and in one clinical trial. There are no previous studies exploring the effect of ulvan on metabolic syndrome or obesity as a whole, and instead the focus has been on hyperlipidaemia (Li et al. 2020; Qi, H et al. 2012) or diabetes (BelHadj et al. 2013). Ulvan consistently reduced triglycerides and LDL-cholesterol and raised HDL-cholesterol, with the greatest efficacy shown at a dose of 125 – 250 mg/kg body weight per day of highly sulphated ulvan (Li et al. 2020; Qi, H et al. 2012). Due to the limited previous studies testing pure ulvan, a study that tested *U. ohnoi* (contains ulvan) in a model of diet-induced metabolic syndrome was included for comparison (Kumar et al. 2015). *U. ohnoi* reduced blood pressure and heart inflammation, fibrosis and stiffness, although it is unclear whether this improvement to cardiovascular function was induced by ulvan or other components of the whole algae (Kumar et al. 2015). Future studies are needed to test ulvan in metabolic syndrome or obesity before clinical trials can be started.

Unlike ulvan, fucoidan has been tested in models of diet-induced obesity and metabolic syndrome (Table 12). In animals, a dose of fucoidan greater than or equal to approximately 200 mg/kg body weight/day decreased body weight and fat mass (Kim, Jeon & Lee 2014; Shang, Song, et al. 2017). A dose of 50 and 100 mg/kg body weight/day was unable to induce weight loss, but did reduce LDL-cholesterol and interestingly reduced HDL-cholesterol as well (Liu et al. 2018). The 100 mg/kg body weight/day dose was sufficient to modulate the gut microbiome towards a less obesogenic profile, as was the 200 mg/kg body weight/day (Liu et al. 2018; Shang, Song, et al. 2017). Currently, fucoidan has produced more health benefits than ulvan, and it remains to be seen whether this is due to structural differences between the two sulphated polysaccharides or whether ulvan will produce similar health benefits when tested in similar models.

Table 11: Ulvan previous studies

Animal Model/Human	Ulvan Dose (Duration)	Effects on Metabolic Syndrome	Mechanisms
Kunming mice (Qi, H et al. 2012) High-cholesterol (2%) diet	125, 250 or 500 mg/kg body weight/day (21 days)	↔ body weight 250 mg best response. ↓ serum triglyceride ↓ LDL cholesterol ↑ HDL cholesterol 500 mg no response.	At 250 mg/kg body weight/day, ulvan with increased sulphation had an increased response 32.8% sulphation compared to 19.5%
Kunming mice (Li et al. 2020) High-cholesterol (2%) diet	125, 250 or 500 mg/kg body weight/day (30 days) Sulphate = 28.1% Total sugar = 65% Uronic acid = 11% Molecular weight = 38.93 kDa	250 mg ↓ body weight 125 and 250 mg ↓ serum triglyceride ↓ LDL cholesterol (125 only) ↑ HDL cholesterol	125 and 250 mg Lipid peroxidation ↓ malondialdehyde Antioxidant enzymes ↑ superoxide dismutase ↑ glutathione peroxidase
Wistar rats (BelHadj et al. 2013) Diabetes induced by alloxan	180 mg/kg body weight/day (30 days)	↓ blood glucose ↑ tolerance to oral glucose, starch and maltose ↓ triglycerides ↓ LDL cholesterol ↑ HDL cholesterol ↓ ALT and AST	Carbohydrate digestion ↓ α-amylase ↓ maltase Lipid digestion ↓ lipase
Wistar rats (Kumar et al. 2015) High-carbohydrate, high-fat diet	*Ulva ohnoi* whole algae – 5% diet (Week 8 – Week 16)	↓ total body fat ↔ body weight ↔ abdominal fat ↓ ALT and AST ↑ NEFA ↔ triglycerides ↔ total cholesterol ↑ glucose tolerance ↑ insulin response ↓ systolic blood pressure ↓ diastolic stiffness ↓ heart inflammation and fibrosis	

Abbreviations used: HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; ALT, alanine transaminase; AST, aspartate aminotransferase; NEFA, non-esterified fatty acids.

Table 12: Fucoidan previous studies

Animal Model/Human	Fucoidan Dose (Duration)	Effects on Metabolic Syndrome	Mechanisms
C57BL/6 mice (Kim, Jeon & Lee 2014) High-fat diet (60%)	1 or 2% diet (4 weeks) No difference in response	↓ body weight ↓ food efficiency ↓ liver weight and steatosis ↓ epididymal fat ↓ triglyceride ↓ LDL-cholesterol ↑ HDL-cholesterol	Lipogenic genes ↓ PPARγ ↓ adipose-specific fatty acid binding protein ↓ acetyl coenzyme A carboxylase Markers of liver damage ↓ glutamic oxaloacetic transaminase ↓ glutamic pyruvic transaminase
C57BL/6J mice (Shang, Song, et al. 2017) High-fat diet (60%)	200 mg/kg body weight/day (16 weeks)	↓ body weight ↓ adiposity index ↓ energy intake ↓ total cholesterol ↓ triglycerides ↓ liver weight ↓ blood glucose ↓ fasting insulin	↓ inflammatory cytokines ↓ lipopolysaccharide binding protein (binds to bacterial lipopolysaccharide – endotoxin) Gut microbiome ↑ Verrucomicrobia ↑ Desulfovibrio ↑ Bacteroides (particularly *Akkermansia*) ↓ *Rikenellaceae* ↓ *Alistipes*
BALB/c mice (Liu et al. 2018) High-fat diet	50 or 100 mg/kg body weight/day (8 weeks)	↔ body weight ↔ epididymal fat ↓ total cholesterol ↓ liver triglycerides 50 mg ↓ LDL-cholesterol ↓ HDL-cholesterol 100 mg ↓ liver cholesterol	Cholesterol synthesis ↓ SREBP2 expression ↓ HMG-CoA reductase (only 100 mg dose) Gut microbiome – 100 mg ↑ Proteobacteria ↓ Firmicutes ↓ *Streptococcus* ↓ *Staphylococcus* ↑ *Alloprevotella*
Overweight or obese adults (Hernández-Corona, Martínez-Abundis & González-Ortiz 2014)	500 mg/day (3 months)	↔ body weight ↔ fat mass ↓ diastolic blood pressure ↔ systolic blood pressure ↓ LDL-cholesterol ↔ HDL-cholesterol ↔ triglycerides ↓ insulin	

Abbreviations used: HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; PPAR, peroxisome proliferator-activated receptor; SREBP2, sterol regulatory element-binding protein 2; HMG-CoA, β-hydroxy β-methylglutaryl-coenzyme A.

Whilst purified phlorotannins and algae peptides will not be the focus of this thesis, they have also been recognised for their potential anti-metabolic syndrome effects although there has been limited studies. The phlorotannins eckol and dieckol both inhibited adipogenesis and increased bone formation *in vitro*, which suggests potential for anti-obesity and anti-osteoarthritis effects (Karadeniz et al. 2015). As discussed earlier, peptides have antioxidant and antihypertensive effects, but they also have potential anti-obesity effects (Fan et al. 2018). Peptides extracted from *Spirulina platensis* inhibited proliferation of preadipocytes *in vitro* and reduced triglyceride production (Fan et al. 2018). Further work is required to determine the mechanism of action, and to test these polyphenols and peptides *in vivo*.

1.8 Thesis Objectives and Hypotheses

There is supporting evidence in the literature that algae contain numerous bioactive compounds that possess therapeutic potential for the symptoms of metabolic syndrome. The Functional Foods Research Group at the University of Southern Queensland has developed a rat model of diet-induced metabolic syndrome to test the effectiveness of functional foods in alleviating symptoms of this condition (Panchal, Poudyal, Iyer, Nazer, Alam, Diwan, Kauter, Sernia, Campbell & Ward 2011). The pathological changes associated with metabolic syndrome are mimicked by feeding rats a high-carbohydrate, high-fat (HCHF) diet (Panchal, Poudyal, Iyer, Nazer, Alam, Diwan, Kauter, Sernia, Campbell & Ward 2011). Control rats fed a corn starch (CS) diet have a lean body type and consistently showed no symptoms of metabolic syndrome.

Using this animal model of diet-induced metabolic syndrome, functional foods such as Queen Garnet plum (Bhaswant et al. 2015), curcumin (Du Preez et al. 2019), purple carrots (Poudyal, Panchal & Brown 2010), coffee extract (Panchal et al. 2012), *Ulva ohnoi* (Kumar et al. 2015) and carrageenan (Du Preez et al. 2020) have been successfully shown to exert positive health benefits in the treatment of metabolic diseases.

The specific objectives of this thesis are listed below:

1. The objectives of this thesis are to determine whether selected bioactive extracts from algae can prevent or attenuate symptoms of metabolic syndrome. The bioactive compounds that will be tested include ulvan, astaxanthin and fucoxanthin.
2. Each bioactive compound is tested at two different doses to observe the dose-response effect on body weight, cardiovascular, liver and metabolic disorders
    a. Ulvan: Low dose - 500 mg/kg body weight. High dose - 2 g/kg body weight
    b. Astaxanthin: Low dose – 0.6 mg/kg body weight. High dose – 1.2 mg/kg body weight
    c. Fucoxanthin: Low dose – 0.65 mg/kg body weight. High dose – 6.5 mg/kg body weight
3. Due to the recent recognition of the role of the gut microbiome in numerous diseases, the changes in body weight, cardiovascular and liver structure and function and metabolic parameters will be correlated with gut microbiome changes to attempt to reveal a mechanism of action.

The hypotheses of this research are listed below:

1. Both the low and high dose of ulvan will induce changes to the gut microbiome and attenuate symptoms of diet-induced metabolic syndrome
2. The high dose astaxanthin will reduce blood pressure, serum lipids and obesity in diet-induced metabolic syndrome. The low dose astaxanthin will provide evidence that a higher dose is necessary to attenuate symptoms of diet-induced metabolic syndrome.
3. Both the low and high dose of fucoxanthin will reduce total fat mass and consequently attenuate symptoms of diet-induced metabolic syndrome
4. Physiological changes will correlate with gut microbiome changes.

1.9 Thesis Outline

Chapter 2: Methods

This chapter contains a detailed recount of the methods used in this research. It begins with the description of the resources used and the compositions of the diets used to induce the lean and obese controls. The experiments conducted whilst the rats were still alive are then explained, followed by a description of the techniques used following euthanasia of the rats.

Chapter 3: Effects of ulvan in diet-induced metabolic syndrome in rats

Ulvan is a soluble fibre extracted from *Ulva ohnoi*, and it was tested in a 16-week study using the model of diet-induced metabolic syndrome to test the effectiveness of a low-dose and a high-dose of ulvan in attenuating symptoms of metabolic syndrome. The changes to the gut microbiome induced by both ulvan and diet are examined in detail in this chapter, followed by a comparison to previous studies in the area.

Chapter 4: Effects of fucoxanthin in diet-induced metabolic syndrome in rats

Fucoxanthin is a carotenoid extracted from *Phaeodactylum tricornutum*, and the results from the 16-week study using the model of diet-induced metabolic syndrome to test the effectiveness of a low-dose and a high-dose of fucoxanthin in attenuating symptoms of metabolic syndrome are presented. Fucoxanthin is delivered in an oleoresin rich in fatty acids also extracted from *P. tricornutum*, in an attempt to increase the bioavailability.

Chapter 5: Low dose astaxanthin is not effective in attenuating symptoms of metabolic syndrome

Astaxanthin is a carotenoid extracted from *Haematococcus pluvialis,* and it was tested in a 16-week study using the model of diet-induced metabolic syndrome to test the effectiveness of a low-dose and a high-dose of astaxanthin in attenuating symptoms of metabolic syndrome. Astaxanthin is delivered in an oleoresin rich in fatty acids also extracted from *H. pluvialis*, in an attempt to increase the bioavailability.

Chapter 6: Conclusions and future recommendations

The major conclusions from the three experimental studies are summarised and then further discussion of how these findings advance our knowledge of ulvan, fucoxanthin and astaxanthin are made. The challenges facing use of algae for therapeutic use and the roadblocks for expansion of the algal industry in Australia are discussed. Finally, future recommendations to address the limitations and gaps in this study are made.

CHAPTER 2 - MATERIALS AND METHODS

The purpose of this chapter is to describe the experimental methods used in this thesis. The rat model of diet-induced metabolic syndrome developed by the Functional Foods Research Group at the University of Southern Queensland was used as the basis for all experimental studies (Panchal, Poudyal, Iyer, Nazer, Alam, Diwan, Kauter, Sernia, Campbell, Ward, et al. 2011). An overview of this model is shown in Figure 17. All experimental protocols were approved by the University of Southern Queensland Animal Ethics Committee under the project approval number 16REA014 (30/9/16 – 30/9/19), under the guidelines of the Australian National Health and Medical Research Council.

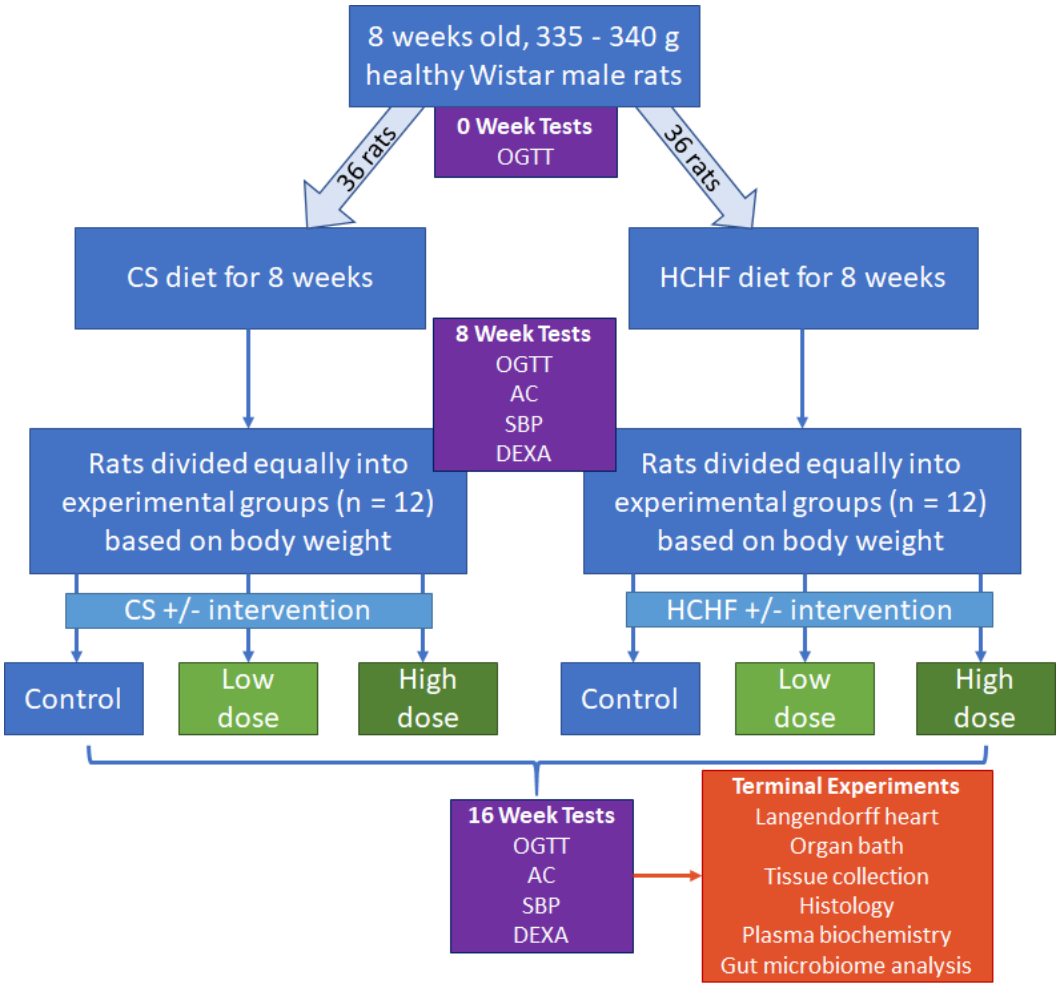


Figure 17: Overall methods flowchart. CS, corn starch diet; HCHF, high-carbohydrate, high-fat diet; OGTT, oral glucose tolerance test; AC, abdominal circumference; SBP, systolic blood pressure; DEXA, dual-energy x-ray absorptiometry

2.1 Resources

2.1.1 Raw materials

Standard laboratory food diet (Specialty Feeds, Glen Forest, WA, Australia) was used during acclimatisation of rats for one week before they were divided into either receiving the corn starch (CS) or high-carbohydrate, high-fat (HCHF) diet. The CS diet was composed of corn starch (Agri Food Ingredients, Kew East, Victoria Australia), powdered rat food (Specialty Feeds, Glen Forest, Western Australia, Australia), Hubble, Mendel, and Wakeman salt mixture (MP Biomedicals LLC, Illkirch, France) and tap water. The HCHF diet was composed of fructose (Tate & Lyle ANZ Pty Ltd, Wacol, Qld, Australia), sweetened condensed milk (Coles, Australia), beef tallow (Carey Brothers Butchers, Warwick), powdered rat food (Specialty Feeds, Glen Forest, Western Australia, Australia), Hubble, Mendel and Wakeman salt mixture (MP Biomedicals LLC, Illkirch, France) and water. Diet composition described in section 2.2 Diet regimes.

Ulva ohnoi, *Phaeodactylum tricornutum* and *Haematococcus pluvialis* were cultivated by PacificBio at a land-based aquaculture facility near Ayr, Queensland, Australia (19°29'S, 147°28E). PacificBio and James Cook University (JCU) extracted the bioactive compounds, ulvan from *U. ohnoi*, fucoxanthin from *P. tricornutum* and astaxanthin from *H. pluvialis*, to be used as interventions in rat models at the University of Southern Queensland (USQ).

Zoletil and lethabarb were sourced from Virbac, Peakhurst, NSW, Australia. When not stated otherwise, chemicals were sourced from Sigma (Sigma Aldrich, Castle Hill, NSW, Australia). Serum samples were collected using serum separator tubes (BD Diagnostics, Scoresby, VIC, Australia).

Tissue samples were fixed in 10% formalin (VWR International, Radnor, Pennsylvania, United States) before being embedded in wax blocks (Paraplast® Regular, melting point 56°C). Paraffin sections were sectioned using a Leica Microtome (Wetzlar, Germany) and collected on to Superfrost™Plus-coated slides (ThermoFischer, Seventeen Mile Rocks, QLD, Australia). Haematoxylin (Sigma Aldrich, Castle Hill, NSW, Australia) was prepared at a concentration of 6.4g/L in a solution of 20% ethanol, 16% glycerol and 0.6%

aluminium persulfate. Eosin Y (Sigma Aldrich, Castle Hill, NSW, Australia) was prepared at a concentration of 10g/L in 90% ethanol. Prior to use, the solution was diluted 1:1 in 90% ethanol. Bouin's solution (Sigma Aldrich, Castle Hill, NSW, Australia) was used as a pretreatment before the Masson's trichrome stain procedure using trichrome stain (Masson) kit (aniline blue solution, biebrich scarlet-acid fuchsin solution, phosphomolybdic acid solution, phosphotungstic acid solution; Sigma Aldrich, Castle Hill, NSW, Australia) and Weigert's iron hematoxylin solution set (solution A and B; Sigma Aldrich, Castle Hill, NSW, Australia). Trichrome stain (Masson) kit and Weigert's iron haematoxylin solution set were prepared as per manufacturer instructions (Sigma Aldrich, Castle Hill, NSW, Australia). After staining, slides were fixed with a coverslip (ThermoFischer, Seventeen Mile Rocks, QLD, Australia) using DPX Mountant for histology (Sigma Aldrich, Castle Hill, NSW, Australia).

2.1.2 Rats

Rats were sourced from the Animal Resource Centre, Murdoch, WA, Australia. Rats were seven weeks old when they arrived and were given approximately one week to acclimatise until they reached 335-340g in body weight. During the acclimatisation, rats were fed a standard laboratory food diet. All rats were housed in individual cages with free access to food and water in a temperature-controlled (21 ± 2ºC) room with an automated 12-hour light and dark cycle. Each rat was supplied with a plastic pipe and wooden stick for environmental enrichment. Individual housing was necessary to determine daily food and water intake.

2.2 Diet regimes

This study used rats where signs of human metabolic syndrome were induced by feeding a diet with increased fructose, condensed milk and beef tallow (high-carbohydrate high-fat or HCHF) with a corn starch diet (CS) serving as the control diet. Preparation and composition of these diets and their physiological responses have been described previously and are shown in the table below (Table 13) (Panchal, Poudyal, Iyer, Nazer, Alam, Diwan, Kauter, Sernia, Campbell, Ward, et al. 2011). The energy densities of CS and HCHF diet diets were 11.23 kJ/g and 17.83 kJ/g, respectively, and an additional 3.85 kJ/mL was added in the fructose-supplemented drinking water for the HCHF diet-fed rats.

Consequently, the HCHF diet can also be considered as a high energy diet in comparison to the CS diet. This means that the effects observed may not be attributed solely to the macronutrient proportions and are likely also influenced by the energy differences. For the treatment diets, an equal proportion of water was replaced with the intervention.

Rats (n = 72, weighing 335 - 340 g) were randomly divided into receiving either the CS or HCHF diet as shown in Figure 17. Further assignment of rats into experimental groups and the timing of the measurements/samples collected are also outlined in Figure 17. Rats were in the protocol for 16 weeks in total, with control rats consuming the same diet for the duration, and intervention rats consuming the same diet as the previous 8 weeks with an additional supplement for weeks 8 - 16.

Table 13: Diet composition components per kg of food

Components (g/kg)	CS	HCHF
Corn starch	570g	-
Fructose	-	175g
Condensed milk	-	395g
Beef tallow	-	200g
Salt mixture	25g	25g
Powdered rat food	155g	155g
Water	250mL	50mL
Drinking water	No additives	25% fructose (w/v)

2.3 Experiments in live rats

Rat body weight and food and water intakes were measured daily on scales (Mettler Toledo, Greifensee, Switzerland) to a precision of 0.1 g to calculate energy intake, feed conversion efficiency and intake of intervention when added to the diet (Panchal, Poudyal, Iyer, Nazer, Alam, Diwan, Kauter, Sernia, Campbell, Ward, et al. 2011). Increase in body

weight (%) was the difference in body weight between 8 – 16 weeks, divided by the body weight at 8 weeks.

2.3.1 Oral glucose tolerance test

The oral glucose tolerance tests (OGTT) were performed after overnight (12 hour) food deprivation at 0, 8 and 16 weeks. The fructose water of HCHF diet-fed rats was replaced with normal water during food deprivation. Basal blood glucose concentrations were determined in tail vein blood using a Medisense Q.I.D glucometer (Abbot Laboratories, Bedford, MA, USA) and glucose test strips (Freestyle Optium Blood Glucose Test Strips, Abbott Diabetes Care Ltd). The rats were then given a glucose load of 2 g/kg body weight by oral gavage of 40% aqueous glucose solution. Following this, blood glucose concentrations were measured after 30, 60, 90 and 120 minutes (Panchal, Poudyal, Iyer, Nazer, Alam, Diwan, Kauter, Sernia, Campbell, Ward, et al. 2011).

2.3.2 Systolic blood pressure

Rats were sedated with Zoletil (10 mg/kg tiletamine, 10 mg/kg zolazepam; Virbac, Peakhurst, NSW, Australia) by intraperitoneal injection for the systolic blood pressure measurement and the dual-energy x-ray absorptiometry (DEXA) scan at 8 and 16 weeks. Systolic blood pressure measurements were performed using an MLT1010 Piezo-Electric Pulse Transducer (ADInstruments) and an inflatable tail-cuff connected to an MLT844 Physiological Pressure Transducer (ADInstruments) connected to a PowerLab data acquisition unit (ADInstruments) (Panchal, Poudyal, Iyer, Nazer, Alam, Diwan, Kauter, Sernia, Campbell, Ward, et al. 2011).

2.3.3 Dual-energy x-ray absorptiometry

The total fat mass, lean mass and bone mineral density were determined by DEXA at 8 and 16 weeks 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.) (Panchal, Poudyal, Iyer, Nazer, Alam, Diwan, Kauter, Sernia, Campbell, Ward, et al. 2011). Fat mass change (g) was calculated by subtracting the 16-week fat mass from the 8-week fat mass for each rat.

2.4 Measurements after euthanasia

Rats were deprived of food for two hours prior to euthanasia by intraperitoneal injection of Lethabarb (100 mg/kg). Following this, the abdominal cavity was opened and ~5 mL blood was collected from the abdominal aorta into serum separator tubes. Blood samples incubated at room temperature for 30 minutes to allow for clotting before being centrifuged at 5,000 × g for 10 minutes. Serum from each rat was stored at -20°C until further analysis. Serum concentrations of total cholesterol and triglycerides, and activities of serum alanine transaminase (ALT) and aspartate transaminase (AST) were commercially determined at The University of Queensland (Gatton) using kits and standards supplied by Olympus (Tokyo, Japan) using an AU 400 Olympus analyser (Panchal, Poudyal, Iyer, Nazer, Alam, Diwan, Kauter, Sernia, Campbell, Ward, et al. 2011).

2.4.1 Tissue weights

The right and left ventricles of the heart were separated after water was circulated through the ventricles to clear blood using a syringe, blotted dry and weighed using scales to a precision of 0.001 g (Mettler Toledo, Greifensee, Switzerland). The liver, kidneys, and retroperitoneal, epididymal and omental fat pads were collected and blotted for weighing. Organ weights were normalised relative to the tibial length at the time of their removal (mg/mm) (Panchal, Poudyal, Iyer, Nazer, Alam, Diwan, Kauter, Sernia, Campbell, Ward, et al. 2011).

2.4.2 Histology

Approximately 5-7 minutes after euthanasia, liver portions for six rats from each group were collected and fixed in 10% neutral buffered formalin for three days. The samples were then dehydrated and embedded in paraffin wax.

Thin sections (~5 µm) of the liver were cut using a Leica Microtome and were collected onto Superfrost™Plus slides. Prior to staining, sections were dewaxed and rehydrated by pre-heating sectioned slides to 60°C for 15 minutes. Three subsequent washes in 100% xylene were used to completely remove paraffin wax. Rehydration was carried out by two

consecutive washes in 100% ethanol, and single washes in 90% ethanol, 70% ethanol and running tap water. All washes were carried out for 2 minutes.

The haematoxylin and eosin stain was used to visualise inflammatory cells and the presence of fat vacuoles in the liver. After dehydration as discussed above, samples underwent the following sequence: haematoxylin (3 minutes), running water (2 minutes), 70% ethanol (2 minutes), eosin Y (7 minutes), 95% ethanol (2 minutes), 100% ethanol (2 minutes, 3 times), and 100% xylene (2 minutes, 3 times).

The extent of collagen deposition was determined in liver sections using Masson's trichrome stain. After dehydration as discussed above, the slides were placed in preheated Bouin's solution (60°C) for 15 minutes. Slides were then cooled and washed to remove the yellow colour. Samples then underwent the following sequence: Weigert's iron haematoxylin solution (5 minutes), running water wash (5 minutes), dip in distilled water (3 times), Beibrich scarlet-acid fuchsin (5 minutes), slides rinsed in distilled water, phosphotungstic acid/ phosphomolybdic acid solution (5 minutes), aniline blue solution (5 minutes), 0.5% acetic acid (1 minute), slides rinsed in distilled water, 95% ethanol (2 minutes), 100% ethanol (2 minutes, 3 times), and 100% xylene (2 minutes, 3 times).

After staining, slides were fixed with a coverslip using DPX Mountant for histology. Liver sections were imaged at 20x magnification using a EVOS FL Color Imaging System (v1.4 (Rev 26059); Advanced Microscopy Group, Bothell, WA, USA) on the standard white light setting. The method used by Goodman (2007), was modified for analysis of these slides. Each liver section was analysed visually by scanning the whole section and a score from 1 – 5 was given to indicate the degree of hepatic fat deposition.

2.5 Gut microbiota analysis

Immediately after euthanasia and organ removal, two or three faecal pellets were collected from the colon of six rats per group and stored at −80 °C in nuclease-free tubes until further analysis. Samples were covered with dry ice pellets to maintain temperature during transport. Gut microbiome analysis was then performed at the University of New South Wales by Priscila Goncalves. Total microbial community DNA was extracted from faecal samples using the DNeasy Powersoil Kit (Qiagen Australia, Chadstone, VIC, Australia)

following the manufacturer's instructions (Zhou, Bruns & Tiedje 1996). The bacterial gut microbiota was then characterised by amplifying and sequencing the 16S rRNA gene. The primers 341F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWG-CAG) and 785R (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTA-CHVGGGTATCTAATCC), both containing illumina overhang nucleotide sequences, were used to amplify the V3-V4 region of the 16S rRNA gene, which was then sequenced on an Illumina MiSeq platform. Sequencing reads were processed to generate operational taxonomic units (OTUs), which were taxonomically classified against the SILVA database (Quast et al. 2012).

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 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 polymerase chain reaction (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 visualised using gel electrophoresis (1% agarose in TAE buffer; w/v), purified using DNA Clean & Concentrator-5 kit (Zymo Research) and quantified using Qubit 3.0 fluorometer (Life Technologies). Paired-end sequencing (2 × 300 base pairs) 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.

For 16S rRNA gene sequencing analysis, sequence data was 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 and removing those shorter than 100 base pairs (Bolger, Lohse & Usadel 2014). USEARCH (version 11.0.667) was used for further processing to merge and quality-filter sequencing reads, excluding reads with < 450 or > 470 nucleotides, in addition to reads with more than one ambiguous base or an expected error higher than 1 (Edgar 2010; Wemheuer & Wemheuer 2017). Filtered sequences were clustered into OTUs at 97% similarity using the UPARSE algorithm implemented in USEARCH (Edgar

2013). Chimeric sequences were removed *de novo* during the clustering step and OTUs were then taxonomically classified (i.e., assigned a likely taxonomic name) by BLASTN (Camacho et al. 2009) against the SILVA database (e-value of 1e-20). All non-bacterial OTUs were removed along with non-BLAST aligned and singletons. Finally, processed sequences were mapped on OTUs 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.

2.6 Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Data from related treatment groups was tested by two-way analysis of variance (ANOVA). When the interaction and/or main effects were significant, the means were compared by Tukey's honestly significant difference (HSD) *post hoc* test. A p value of <0.05 was considered as statistically significant. All statistical analyses were performed using RStudio version 1.2.1335 (R Foundation for Statistical Computing, Vienna, Austria).

Multivariate statistical analysis of the bacterial community structure across groups was performed using the R package mvabund (Wang, Y et al. 2012). Generalised linear models (negative binomial) were generated incorporating library size (ie, total number of sequences per sample; log-transformed) as offset. *Post hoc* tests then identified the OTUs that were affected by diet, treatment, or by both factors (ie, differentially abundant OTUs). Due to the substantial differences in sequencing coverage between samples, the dataset was normalised based on library sizes using DESeq2 (Love, Huber & Anders 2014). The normalised sequencing dataset was used for distance-based (principal coordinates analysis, non-metric multidimensional scaling, and permutational multivariate analysis of variance with Bray-Curtis dissimilarity) and correlation analyses as well as for plots of taxonomic composition. Differences in bacterial structure between groups were also explored by calculating alpha-diversity metrics (richness, Chao1, Shannon, and Inverse Simpson indices) and the ratio of Firmicutes/Bacteriodetes (F/B). The effect of diet, treatment, and their interaction on alpha-diversity indices and F/B ratios were evaluated by two-way ANOVA, followed by the Tukey multiple comparison post hoc test.

CHAPTER 3 – EFFECTS OF ULVAN IN DIET-INDUCED METABOLIC SYNDROME IN RATS

3.1 Introduction

Ulvan is a soluble fibre component of the prolific green seaweed *Ulva*, an edible sea lettuce well-known for the formation of "green tides" owing to rapid growth rates and broad environmental tolerance (Zhao, J et al. 2018). These green tides are abnormal proliferations of *Ulva*, that in extreme cases can cover an area of 20 000 km^2 and persist for between 76 – 110 days (Liu, Wang & Zhang 2016). Green tides have been a yearly occurrence in the Yellow Sea since 2007, causing damage to the coastal landscape, mariculture and local ecosystems and consequently necessitating costly removal by the Chinese government (Li et al. 2018). Whilst this is problematic, this collected *Ulva* biomass could be repurposed for human food or animal feed products to recover and perhaps exceed the expense of removal (Li et al, 2018). Further, it demonstrates that some samples of *Ulva* are highly suitable for cultivation due to the potential for rapid growth (Zhao, J et al. 2018). The heavy metal and pesticide content of *Ulva* from these green tides was tested over two years, and determined to be safe for human consumption (Li et al. 2018). However, the heavy metal and pesticide content is heavily dependent on the algae growing conditions and does not indicate that all *Ulva* is safe for human consumption.

Ulva is consumed in Japan and Korea in both fresh (e.g. salads) or dried (e.g. aonori) forms but has not been adopted within Western countries despite worldwide distribution of the seaweed (Cherry et al. 2019). The nutritional content of *Ulva* varies between species and growing conditions, with aquaculture-grown *Ulva ohnoi* containing 19% protein and 29% fibre (Glasson et al. 2017). Unique to the genus *Ulva* is the soluble fibre ulvan, which comprises 12% of the dry biomass (Glasson et al. 2017). Ulvan is a sulphated heteropolysaccharide composed of variable amounts of uronic acids, including glucuronic and iduronic acids, alternating with neutral sugar moieties, such as rhamnose, xylose and glucose (Figure 18) (Alves, Sousa & Reis 2013).

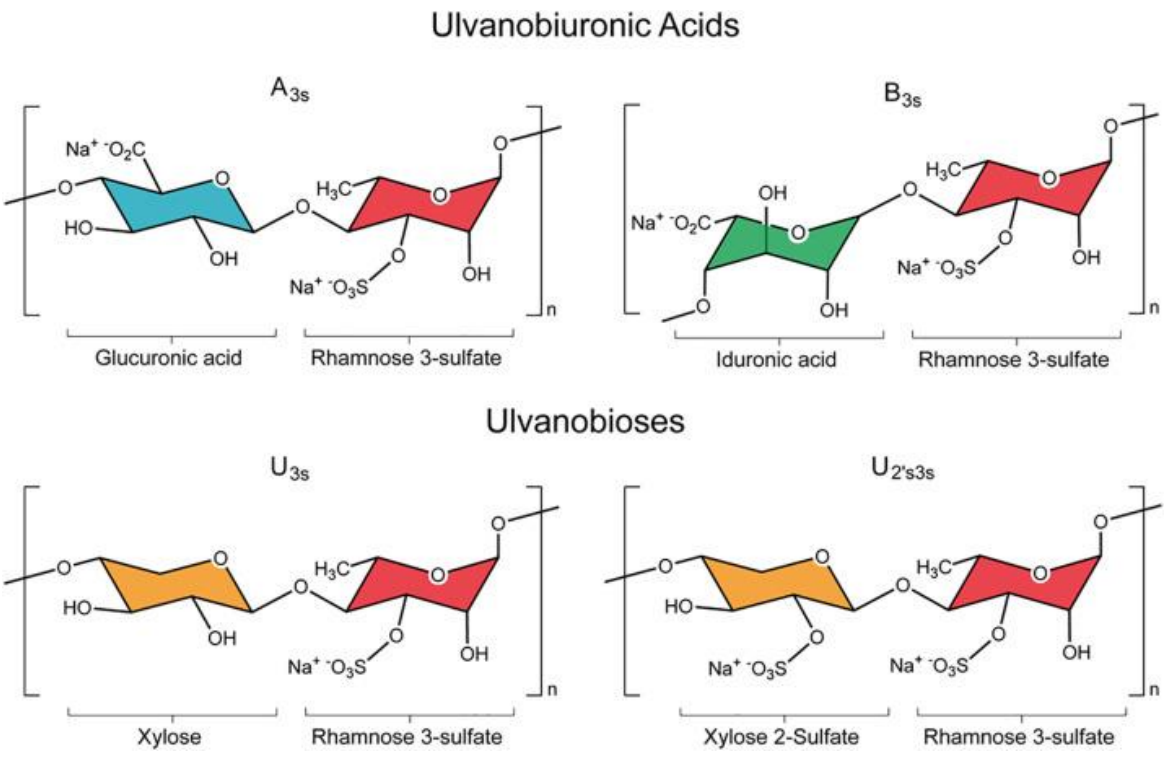


Figure 18: Nomenclature and structure of the major repeating disaccharide units that comprise ulvan (Kidgell et al. 2019)

In Australia, only 28% of adults meet the adequate dietary fibre requirements of 25-30 g/day, and the values reported in the 2011-12 National Nutrition and Activity survey determined that the mean fibre intake for adults was just 20.7g/day (Fayet-Moore et al. 2018). Fibre intake in Australia could be increased by increasing consumption of wholegrain, fruits and vegetables (Fayet-Moore et al. 2018). An alternative worth considering is increasing the fibre content of other commonly consumed foods. *Ulva* is then a potential public health intervention to improve fibre intake. Assuming a fibre content of 29% in *Ulva*, a daily consumption of roughly 35 g of *Ulva* seaweed would provide 10 g of fibre. This is considerably higher than the daily seaweed consumption in Japan, roughly 5 g/day, and could represent a barrier in Western countries where seaweed intake is minimal (Zava & Zava 2011). This could be overcome by instead extracting the fibre from ulvan and incorporating it into other food products regularly consumed in Australia such as bread or pasta.

Raising fibre intake is associated with improved glycaemic control in diabetic patients, mitigation of chronic inflammation, lowered cholesterol and blood pressure and modulation of the gut microbiota towards a 'healthier' profile (Dahl et al. 2017). Administration of *Ulva ohnoi* or *Ulva linza* to rodents reduced body fat, abdominal fat and systolic blood pressure while improving liver function (Kumar et al. 2015; Ramirez-Higuera et al. 2014). However, the responses to ulvan have varied, likely due to method of ulvan extraction and environmental condition during algae growth or seasonality (Alves, Sousa & Reis 2013). Ulvan possesses antioxidant, antihyperlipidaemic, anticancer and antiviral properties, and is a soluble fibre resistant to enzymatic digestion suggesting that it could exert prebiotic effects (Kidgell et al. 2019). Ulvan extracted from several *Ulva* species improved the blood lipid profile and liver function, but it was unable to reduce body weight in rodents (BelHadj et al. 2013; Hassan et al. 2011; Qi & Sheng 2015).

The present study used a 16-week rat model of diet-induced metabolic syndrome (Panchal, Poudyal, Iyer, Nazer, Alam, Diwan, Kauter, Sernia, Campbell, Ward, et al. 2011) to determine whether ulvan can reduce obesity, cardiovascular damage or liver changes by possible modulation of gut microbiota. This model was previously used to test the whole algae *Ulva ohnoi* at a dose of 5% diet, so an ulvan dose of 2% diet based on the approximately 40% fibre content in the whole algae was selected to compare these health responses (Kumar et al. 2015). Rats in this model were initially fed a HCHF diet for the first eight weeks before ulvan was added to this diet for the last eight weeks. CS diet was used as the control diet for the study (Panchal, Poudyal, Iyer, Nazer, Alam, Diwan, Kauter, Sernia, Campbell, Ward, et al. 2011). The hypothesis for this first study was that ulvan would reverse metabolic, cardiovascular and liver changes in diet-induced metabolic syndrome. It was predicted that the major mechanism of these effects would be a prebiotic effect of ulvan to modulate the gut microbiota. Further, in a second study, a lower ulvan dose of 0.5% was tested in the same model. The purpose of this second study was to examine whether a dose that could be converted to a suitable human dose could provide these same health benefits.

3.2 Methods and Materials

The materials and methods are conducted as described in CHAPTER 2 - MATERIALS AND METHODS with the following modifications.

3.2.1 Production and analysis of ulvan

Biomass of *Ulva ohnoi* (Gen-bank accession number KF195509, strain JCU 1 for ulvan extraction) was cultivated by PacificBio at a land-based aquaculture facility near Ayr, Queensland, Australia (19°29'S, 147°28E) (Figure 19) (Lawton et al. 2013).

Figure 19: Ulva ohnoi grown at PacificBio facility near Ayr, north Queensland (PacificBio 2020)

Ulvan was extracted and characterised by Marinova Pty. Ltd. (Cambridge, Tasmania, Australia), using a proprietary aqueous extraction process and certified protocols, and supplied by PacificBio. The content of heavy metals was determined by ICP-MS at the Advanced Analytical Centre of James Cook University. The average molecular weight

was determined by Christopher Glasson at James Cook University by dissolving ulvan (4 mg) in eluent (50 mM $NaNO_3$ and 0.02% NaN_3) and then filtering with a 0.22 μm syringe filter. Sample solutions were analysed with a high-performance size exclusion chromatography system (1260 Infinity II LC GPC/SEC system, Agilent Technologies Australia, Mulgrave, Victoria, Australia) fitted with a 50 x 7.5 mm PL aquagel-OH Guard coupled to a 7.5 x 300 mm PL aquagel-OH Mixed-H column. Each sample (5 μL) was injected at 45°C at a flow rate of 0.7 ml/min. The eluted fractions were detected by a refractive index and UV spectrophotometer (280 nm). Molecular weight (Mn = 1.4-1,194 g/mol) was calculated using Agilent GPC/SEC software against polyethylene glycol standards.

3.2.2 Diet regimes

Rats (n = 72, weighing 338 ± 0.4 g) were randomly distributed into six experimental groups (n = 12 rats/group) as shown in Table 1. The lower ulvan dose was selected based on previous literature that 250 mg ulvan/kg/day was optimal in rats (Qi & Sheng 2015). The higher dose was selected to test the upper limits of the dose-response curve to ulvan as a comparable portion of ulvan to a previous study (*Ulva ohnoi* whole dried biomass at 5% diet inclusion) that had additional health benefits to the lower dose studies (Kumar et al. 2015). For the treatment diets, an equal proportion of water was replaced with ulvan.

Table 14: Treatment groups diet regime

Treatment Group	0 – 8 Week Diet	8 – 16 Week Diet
C	CS	CS
CUL	CS	CS + 0.5 % ulvan
CUH	CS	CS + 2 % ulvan
H	HCHF	HCHF
HUL	HCHF	HCHF + 0.5 % ulvan
HUH	HCHF	HCHF + 2 % ulvan

3.2.3 Short chain fatty acid measurements

The contents of the caecum were collected during dissection and then stored at –20°C prior to short-chain fatty acid analysis. Caecum samples were prepared following the acidification method with the following adjustments (Zhao, Nyman & Jonsson 2006). Approximately 300 mg of caecum content was weighed out to 1 mg precision, thawed and suspended in 3 mL of Milli-Q water. This caecum suspension was acidified to a pH of 2-2.5 by adding 5M HCl and then sonicated in an ultrasonic bath for three minutes. Due to the HCHF diet, the supernatant after centrifugation was turbid and PES Express 0.22µm Millipore syringe filters were used for all samples to correct this. It was confirmed that there was no visible difference to the GCMS short-chain fatty acid peaks after using these syringe filters.

Chromatographic analysis was carried out using an Agilent 7890A GC system equipped with a flame ionisation detector and a 7693 autosampler (Agilent, USA). A DB-Wax Ultra Inert GS column (Agilent, USA) was used. The initial oven temperature was 100°C and the temperature was ramped up 10°C every minute until 210°C was reached, with a solvent delay of four minutes. The injection volume was 1 ml. A Milli-Q blank was run between each sample to ensure there was no carry-over. A mixed standard (Volatile Free Fatty Acid Mix, TraceCERT) was used to create a dilution curve (seven dilutions 0.15mL – 3mL) to determine the short-chain fatty acid concentrations in the caecum samples (mmol/L) (Zhao, Nyman & Jonsson 2006). This concentration was then used to calculate the number of mmol in the sample, and that was divided by the wet weight of the caecum content used to obtain the short chain fatty acid concentration in mmol/g.

3.2.4 Gut microbiome measurements

Ulvan low dose samples were analysed as per section Chapter 2 – Materials and Methods.

3.2.4.1 Ulvan high dose gut microbiome extraction

Immediately following organ removal, two to three faecal pellets were collected from the colon of rats and stored at –80°C in nuclease-free tubes (n = 6 rats/group). DNA extraction and 16S rRNA gene sequencing were performed by the Australian Genome Research Facility, Brisbane, QLD, Australia. The V1-V3 region of the 16S rRNA gene was selected for amplification. The primers used were 27F (5′-AGAGTTTGATCMTGGCTCAG-3′)

and 519R (5′-GATTACCGCGGCKGCTG-3′). PCR amplicons were generated using an AmpliTaq Gold 360 Master Mix (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). The concentrations of the resulting amplicons were measured by fluorometry (Invitrogen Picogreen, Mount Waverley, VIC, Australia) and normalised. The concentration of the equimolar pool was then measured by qPCR (KAPA) followed by sequencing on the Illumina MiSeq (Illumina Inc, San Diego, CA) with 2 × 300 bp paired-end chemistry. Sequencing data were analysed using in-house pipeline employing UPARSE (implemented in USEARCH(Edgar 2010)) for clustering into operational taxonomic units (OTUs) at 97% sequence similarity and SILVA database for taxonomy assignment (version 132)(Edgar 2013).

For 16S rRNA gene sequencing analysis, sequence data was initially quality-filtered and trimmed using Trimmomatic (version 0.36) truncating reads if the quality dropped below 10 in a sliding window of 4 bp and removing those shorter than 36 bp (Bolger, Lohse & Usadel 2014). USEARCH (version 9.2.64) was used for further processing to merge and quality-filter sequencing reads, excluding reads with < 450 or > 550 nucleotides, in addition to reads with more than one ambiguous base or an expected error higher than 1 (Edgar 2010; Wemheuer & Wemheuer 2017). Filtered sequences were clustered into OTUs at 97% similarity using the UPARSE algorithm implemented in USEARCH (Edgar 2013). Chimeric sequences were removed *de novo* during the clustering step and OTUs were then taxonomically classified (i.e., assigned a likely taxonomic name) using the SILVA database (-usearch_global command at 97% identity; SILVA database version 132). All non-bacterial OTUs were removed along with unassigned sequences and singletons. Finally, processed sequences were mapped on OTUs 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.

3.2.5 Statistical analysis

Statistical methods were conducted as described in section Chapter 2.6 Statistical analysis with the following additions. The high and low dose studies were analysed separately as they were conducted at different timepoints. The association between physiological

parameters and bacterial community was investigated using the Mantel test (R package vegan) (Oksanen et al.). Vector fitting to ordinations (functions envfit and bioenv from the R package vegan) was used to identify the physiological variables that best predicted bacterial community composition. Physiological variables with significant impact on the bacterial community ordination were then correlated with the differentially abundant OTUs using Pearson correlation (R package Hmisc), in order to identify the physiological variables with positive or negative relationships to individual OTUs (Harrell Jr & Dupont 2017). A p value of <0.05 was considered as statistically significant.

3.3 Results

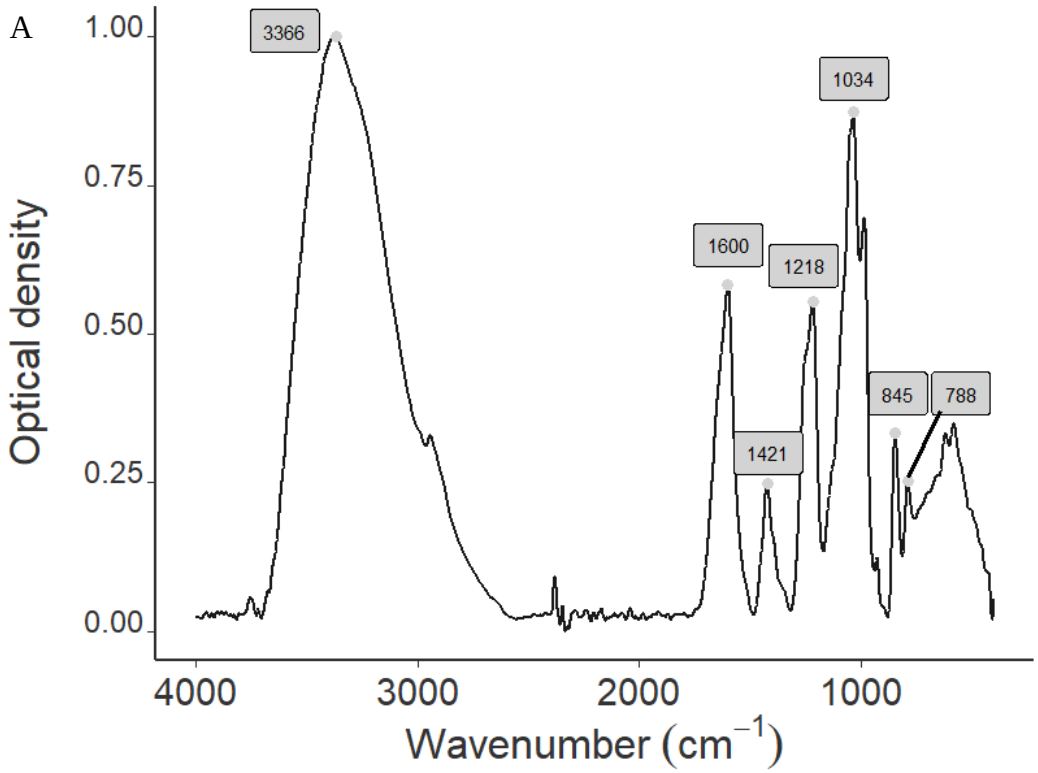
3.3.1 Ulvan composition

Purity of the ulvan extract was determined to be 86.7%. The average molecular weight was 215.4 kDa with a polydispersity index of 3.7. The neutral sugar profile of the ulvan extract was comprised primarily of rhamnose (Table 15). Potassium was found in the highest proportions of the detectable cations (Table 15). A Fourier transform infrared spectrum (FTIR) of the ulvan extract showed strong absorbances at 3366, 1600, 1218 and 1034 cm^{-1} and smaller peaks at 1421, 845 and 788 cm^{-1} (Figure 20). These peaks were identified and are shown in Figure 20B.

Table 15: Ulvan analysis

Extract properties (% w/w)	
Neutral carbohydrates	27.0
Uronic acids	21.0
Sulfate	20.7
Cations	11.6
Neutral sugar profile (mol %)	
Rhamnose	81.30
Xylose	9.63
Galactose	3.38
Glucose	2.72
Mannose	1.25
Fucose	1.13
Arabinose	0.60
Cations (% w/w)	
Potassium	8.05
Sodium	1.81
Magnesium	1.54
Calcium	0.26
Heavy metals (ppm)	
Arsenic	0.59
Cadmium	0.06
Mercury	1.89
Lead	1.41

A



B

Absorbance (cm^{-1})	Assignment	Functional Group/Sugar
3366	OH	Hydroxyl groups / monosaccharides
1600	C=O ν_{asym}	Carboxyl groups / uronic acids
1421	C=O ν_{sym}	Carboxyl groups / uronic acids
1218	S=O ν_{asym}	Sulfonyl group / sulfate esters
1034	C-O-C	Glycosidic linkage / monosaccharides
845	C-O-S	Sulfate ester
788	C-O-S	Sulfate ester

*Figure 20: Fourier transform infrared (FTIR) spectra of ulvan **A** FTIR spectrum **B** FTIR spectrum major peak assignments*

3.3.2 High dose ulvan

3.3.2.1 Body composition, diet intake and metabolic changes

The measurements at 8 weeks confirmed that HCHF diet-fed rats had a higher body weight and fat mass compared to CS diet-fed rats (Figure 21A and 21B). The difference in body weight between the two diets continued to increase in the following 8 weeks of the study. Ulvan suppressed body weight gain and total fat mass increase in HUH compared to H rats (Figure 21B and 21C). In the CS diet-fed groups, there was no difference between CUH and C for both body weight and fat mass. Whilst there was a decrease in total fat mass, the abdominal fat pads were unchanged in HUH rats compared to H rats ($p = 0.1$, Figure 21D).

Ulvan intake did not change food or water intake in HCHF diet-fed rats (Table 16), instead reducing feed conversion efficiency of HUH rats compared to H rats (Figure 21E). In CS-diet fed groups, ulvan reduced food intake and total energy intake compared to C rats (Table 16). The CUH rats consumed approximately double the intake of ulvan of HUH rats due to the increased intake of food by CS diet-fed rats in comparison to HCHF diet-fed rats. After 16 weeks, HCHF diet-fed rats had a higher fasting blood glucose concentration and reduced tolerance to an oral glucose load than C rats (Table 16). Ulvan intake did not change fasting glucose concentrations nor glucose loading response. Total plasma cholesterol and triglycerides were also unchanged by ulvan intake (Table 16). Serum ALT activities were increased in H rats compared to C and CUH, and HUH rats were intermediate to both H and C (H and HUH $p = 0.058$). All groups fed HCHF diet had a higher systolic blood pressure than CS diet-fed rats (Table 16). Ulvan did not change systolic blood pressure.

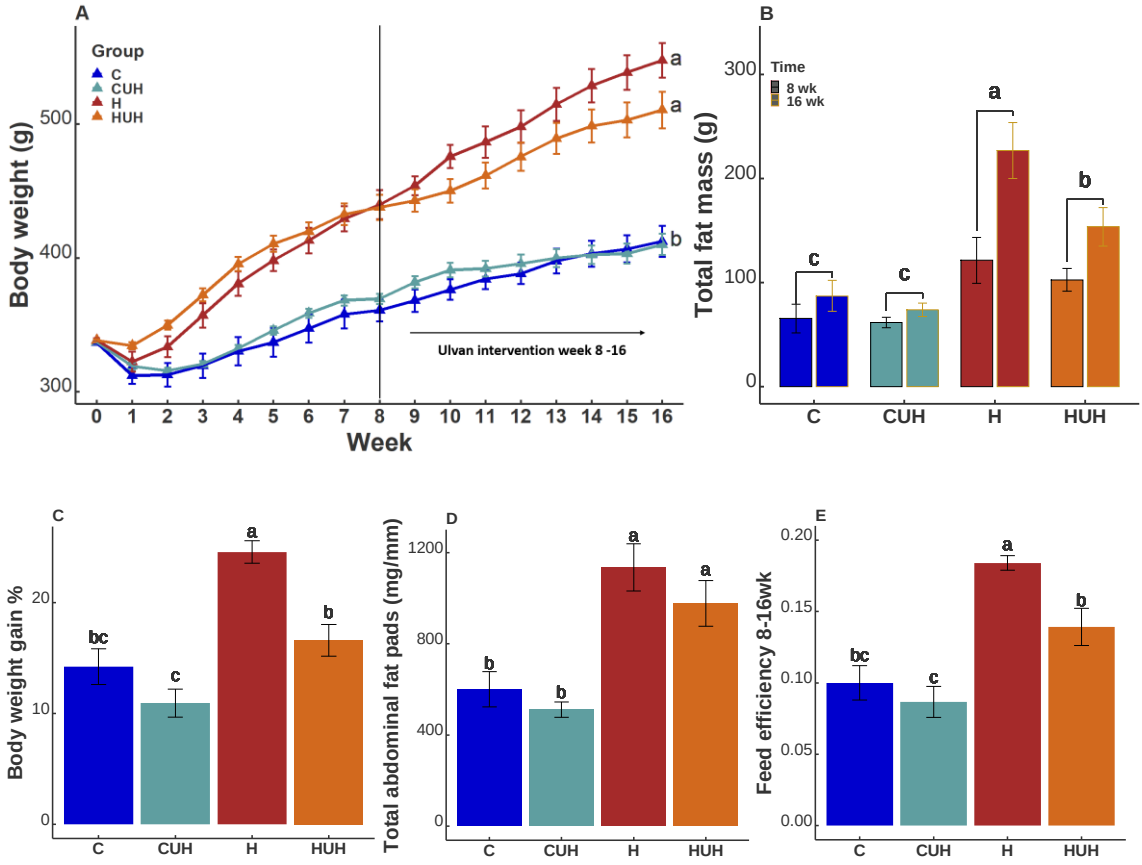


Figure 21: Body composition and diet intake. ***A*** *Body weight 0-16 weeks.* ***B*** *Average change in fat mass from 8-16 weeks.* ***C*** *Body weight gain % from 8-16 weeks. D Abdominal fat content mg tissue weight/mm tibial length.* ***E*** *Average daily feed conversion efficiency 8-16 weeks. Values are mean ± SEM, n = 6-12. Means without a common letter differ, p < 0.05. C, CS diet-fed rats; CUH, CS diet-fed rats supplemented with 2% ulvan; H, HCHF diet-fed rats; HUH, HCHF diet-fed rats supplemented with 2% ulvan.*

Table 16: Physiological parameters in rats with high ulvan dose

Variable	C	CUH	H	HUH	Two Way Anova		
					Diet	Treatment	Interac-tion
Ulvan intake g/ kg body weight/day	0 ± 0.0^{c}	2.07 ± 0.03^{a}	0 ± 0.0^{c}	0.97 ± 0.03^{b}	< 0.001	< 0.001	< 0.001
Body weight (g) week 8	361 ± 8^{b}	369 ± 4^{b}	440 ± 11^{a}	438 ± 10^{a}	< 0.001	0.716	0.555
Body weight (g) week 16	412 ± 12^{b}	410 ± 8^{b}	548 ± 13^{a}	511 ± 14^{a}	< 0.001	0.135	0.186
Water intake (g/day) week 0-8	30.9 ± 2.0	32.9 ± 1.8	29.0 ± 1.4	32.3 ± 0.8	0.501	0.112	0.710
Water intake (g/day) week 8-16	26.1 ± 2.4	28.6 ± 1.7	29.2 ± 1.6	29.7 ± 0.6	0.265	0.362	0.551
Food intake (g/day) week 0-8	43.6 ± 1.0^{a}	44.6 ± 0.5^{a}	27.1 ± 0.8^{b}	26.7 ± 0.6^{b}	< 0.001	0.641	0.339
Food intake (g/day) week 8-16	45.6 ± 1.4^{a}	41.4 ± 0.6^{b}	26.0 ± 0.5^{c}	24.3 ± 0.6^{c}	< 0.001	< 0.001	0.125
Energy intake (kJ/day) week 0-8	519 ± 17^{b}	501 ± 5^{b}	607 ± 19^{a}	601 ± 10^{a}	< 0.001	0.342	0.637
Energy intake (kJ/day) week 8-16	519 ± 17^{b}	464 ± 7^{c}	581 ± 18^{a}	547 ± 12^{a}	< 0.001	0.002	0.438
Abdominal circumference week 8 (cm)	19.0 ± 0.4^{b}	19.0 ± 0.1^{b}	20.5 ± 0.5^{a}	20.5 ± 0.3^{a}	< 0.001	0.749	0.897
Abdominal circumference week 16 (cm)	19.5 ± 0.5^{b}	20 ± 0.5^{b}	24 ± 0.5^{a}	22 ± 0.5^{a}	< 0.001	0.173	0.064
Lean mass week 8 (g)	284 ± 4^{c}	298 ± 4^{bc}	309 ± 11^{ab}	324 ± 9^{a}	0.002	0.063	0.940
Lean mass week 16 (g)	300 ± 12	308 ± 6	302 ± 18	317 ± 12	0.630	0.346	0.794
Fat mass week 8 (g)	65 ± 14^{bc}	62 ± 5^{c}	121 ± 22^{a}	103 ± 11^{ab}	< 0.001	0.364	0.556
Fat mass week 16 (g)	87 ± 15^{c}	74 ± 7^{c}	227 ± 27^{a}	154 ± 19^{b}	< 0.001	0.016	0.077
SBP week 16 (mmHg)	125 ± 6.7^{b}	125 ± 1^{b}	149 ± 4^{a}	152 ± 4^{a}	< 0.001	0.837	0.756

Table 16: Physiological parameters in rats with high ulvan dose continued

Variable	C	CUH	H	HUH	Two Way Anova		
					Diet	Treatment	Interaction
Tissue wet weight (mg/mm tibial length)							
Kidney	48.8 ± 1.1^{b}	47.5 ± 0.9^{b}	61.3 ± 2.2^{a}	60.4 ± 1.5^{a}	< 0.001	0.462	0.922
Omental fat	187 ± 18.7^{b}	159 ± 11.2^{b}	307 ± 29.3^{a}	249 ± 23.5^{ab}	< 0.001	0.0611	0.5119
Epididymal fat	117 ± 24.3^{b}	104 ± 9.3^{b}	209 ± 29.4^{a}	216 ± 24.1^{a}	< 0.001	0.903	0.661
Retroperitoneal fat	296 ± 35.9^{b}	248 ± 16.3^{b}	619 ± 56.6^{a}	512 ± 55.8^{a}	< 0.001	0.114	0.538
Liver	230 ± 10.3^{b}	237 ± 5.5^{b}	349 ± 5.5^{a}	364 ± 2.5^{a}	< 0.001	0.312	0.676
Left ventricle and septum	23.8 ± 0.6^{ab}	21.8 ± 0.8^{b}	26.9 ± 1.4^{a}	24.2 ± 1.1^{ab}	0.015	0.041	0.774
Right ventricle	4.9 ± 0.9	4.2 ± 0.2	5.6 ± 0.4	5.0 ± 0.2	0.057	0.126	0.805
Serum parameters							
Total cholesterol (mmol/L)	1.6 ± 0.09	1.5 ± 0.04	1.6 ± 0.02	1.7 ± 0.07	0.148	0.765	0.291
Triglycerides (mmol/L)	0.78 ± 0.05^{ab}	0.48 ± 0.06^{b}	1.31 ± 0.16^{a}	1.14 ± 0.17^{a}	< 0.001	0.100	0.668
ALT (U/L)	32.0 ± 2.7^{bc}	25.6 ± 1.6^{c}	43.2 ± 3.3^{a}	34 ± 2.1^{ab}	< 0.001	0.003	0.600
AST (U/L)	83.2 ± 4.5	86.8 ± 4.0	107.6 ± 4.8	92.2 ± 6.0	0.048	0.370	0.111
Oral glucose tolerance test							
AUC week 8 (mmol/L x min)	554 ± 29^{ab}	515 ± 20^{b}	545 ± 17^{ab}	605 ± 12^{a}	0.005	0.594	0.021
AUC week 16 (mmol/L x min)	523 ± 29^{bc}	499 ± 13^{c}	570 ± 19^{ab}	590 ± 11^{a}	< 0.001	0.891	0.171

SBP, systolic blood pressure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the curve; C, CS diet-fed rats; CUH, CS diet-fed rats supplemented with 2% ulvan; H, HCHF diet-fed rats; HUH, HCHF diet-fed rats supplemented with 2% ulvan.

3.3.2.2 Liver changes

The total liver wet weight was higher in H rats than C rats, but there was no change in ulvan treatment groups in comparison to controls (Table 16). Livers from H rats showed increased fat deposition causing hepatocyte ballooning, inflammatory cell infiltration, and a higher incidence of collagen deposition in zone 2 areas (away from central and portal veins) than C rats (Figure 22). In the four rats per group that were analysed, the average score of liver fat deposition out of a maximum of 5 was 0.5 (C), 0 (CUH), 4 (H) and 2.75 (HFH). Hence, liver fat deposition was lower for HUH compared to H.

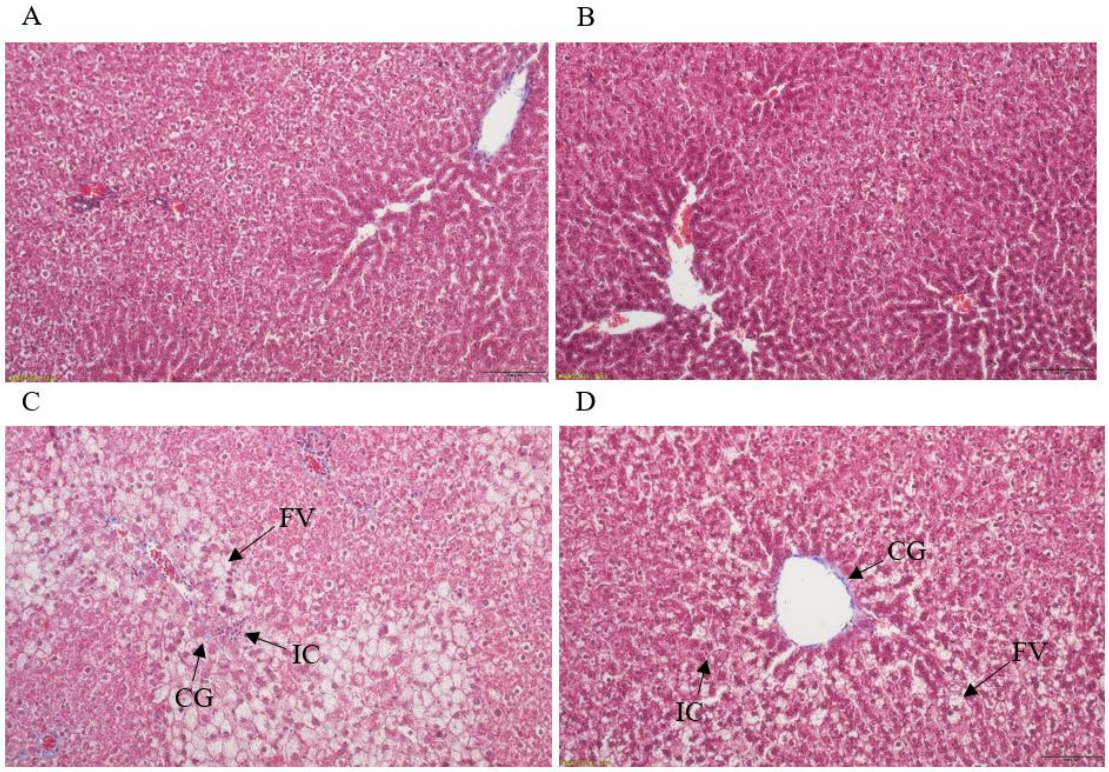


Figure 22: Masson's staining of the liver to show fat deposition and collagen. Fat vacuoles marked as 'FV', inflammatory cells are marked as 'IC' and collagen is marked as 'CG', n = 6. Scale = 100 µm. ***A*** *C liver.* ***B*** *CUH liver.* ***C*** *H liver.* ***D*** *HUH liver. C, corn starch diet-fed rats; CUH, CS diet-fed rats supplemented with 2% ulvan; H, HCHF diet-fed rats; HUH, HCHF diet-fed rats supplemented with 2% ulvan. An enlarged image of H and HUH liver can be found in Supplementary Figure 1.*

3.3.2.3 Microbiome analysis

Distance-based multivariate analysis combining the abundances of all OTUs (n = 349) showed that each of the treatments produced a distinct bacterial composition. Ordinate separation is more evident between groups that received different diets (C/CUH in relation to H/HUH) (Figure 23A). PERMANOVA showed that both diet ($p = 0.001$) and ulvan supplementation ($p = 0.043$) have a statistically significant effect on the gut microbial community (but not their interaction; $p = 0.239$). Post-hoc pairwise comparisons revealed that all groups have unique bacterial communities ($0.004 < p < 0.014$). The Bray-Curtis dissimilarity matrices stress value was 0.121 for this non-metric multidimensional scaling plot, indicating that the distance between the samples in reduced dimensional space correlates well with the actual multivariate distance of the samples.

Distance-based multivariate analyses combining data from 27 physiological parameters measured at week 16 showed that ordinate separation was more evident between groups that received different diets (C/CUH in relation to H/HUH) (Figure 23B). PERMANOVA showed that both diet and supplement (but not their interaction) had a statistically significant effect on rat physiological performance (diet $p = 0.001$, ulvan $p = 0.046$, interaction $p = 0.542$). Post-hoc pairwise comparisons showed that the physiological response between C and CUH ($p = 0.210$) and H and HUH ($p = 0.291$) were not statistically different. The Euclidean distance stress value was 0.02, which indicates that the distance between samples on the multidimensional scaling plot is an almost perfect representation of distance (perfect is zero).

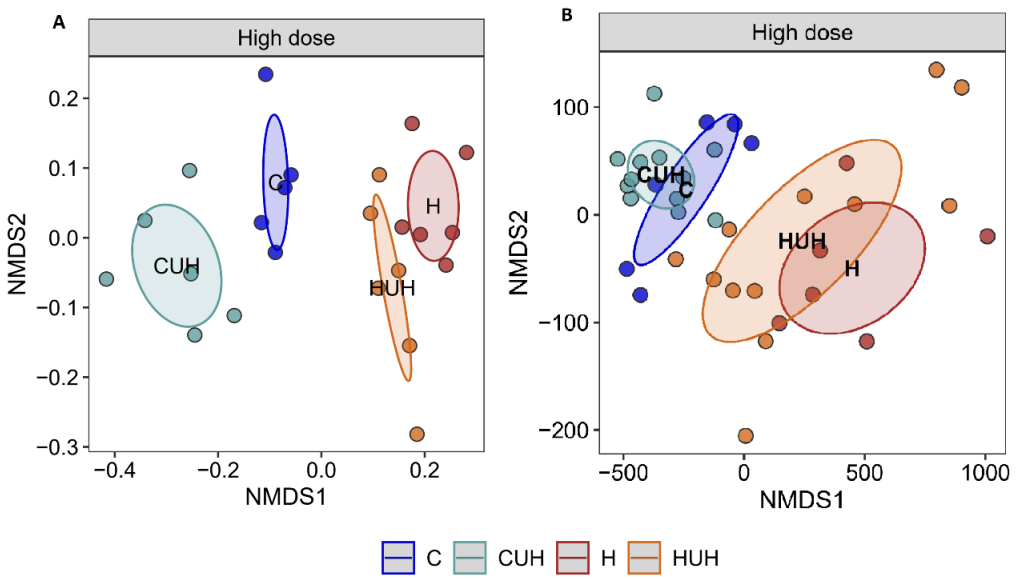


Figure 23: NMDS plots include data measured at week 16. In these plots, each point corresponds to one sample (n = 6-12 per treatment) and incorporates the response of 27 physiological parameters. ***A*** *Bacterial community structure of rat guts under different diets supplemented with ulvan. Non-metric multidimensional scaling (NMDS) plots incorporating all OTUs generated (n = 349). In these plots, each point corresponds to one sample and incorporates the abundance of all OTUs present in that sample.* ***B*** *Physiological response of rats subjected to dietary supplementation ulvan.*

Diet had a stronger effect on the bacterial community composition, affecting the abundance of 130 OTUs (37% of the gut microbiota), while the abundance of 78 OTUs was significantly affected by the supplementation with high dose ulvan, and the combination of diet and supplement had an effect on the abundance of 28 OTUs (Supplementary Table 1). Analysis of bacterial community structure across treatments showed that bacteria from the phylum Firmicutes were the most abundant, regardless of diet or ulvan supplementation (Figure 24A). The relative abundance of bacteria from the phylum Bacteroidetes was reduced by HCHF diet ($p = 0.044$) and increased by ulvan intake ($p = 0.0002$). In addition, the relative abundance of bacteria from the phylum Verrucomicrobia was reduced by the ulvan supplementation ($p = 0.021$). This difference in bacterial composition was further shown by calculating the Firmicutes/Bacteroidetes (F/B) ratio, which was shown to be strongly impacted by diet ($p = 0.006$) (Figure 24B). Ulvan supplementation in both diets (CUH and HUH) reduced this F/B ratio ($p = > 0.001$).

The gut microbiota richness was reduced by the HCHF diet compared to CS diet. Ulvan intake did not change microbiota richness or diversity (Figure 25).

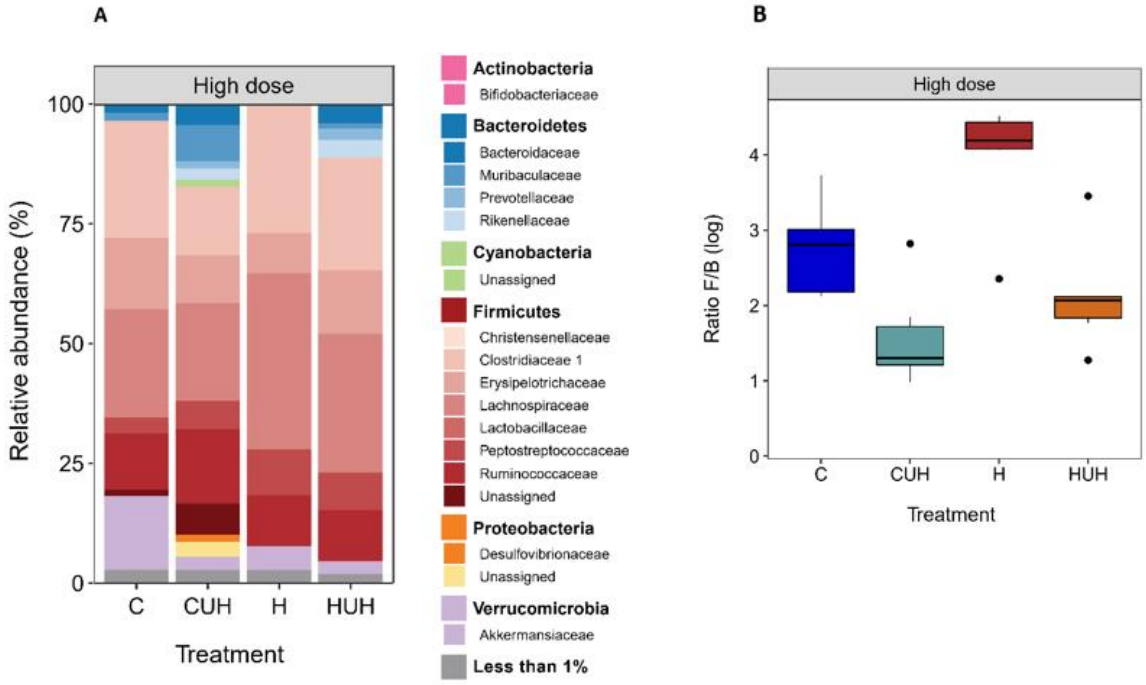


Figure 24: Bacterial taxonomic composition of rat faecal samples. ***A*** *Gut bacterial composition in rats fed control starch (C) and high carbohydrate and high fat diet (H) diets, supplemented with ulvan. Plot displays bacterial communities at the phylum (in bold, main colours) and family levels, and include sequences with relative abundance higher than 1% (filtering performed at the family level).* ***B*** *Ratio of Firmicutes and Bacteroidetes abundances in the gut microbiota. C, CS diet-fed rats; CUH, CS diet-fed rats supplemented with 2% ulvan; H, HCHF diet-fed rats; HUH, HCHF diet-fed rats supplemented with 2% ulvan.*

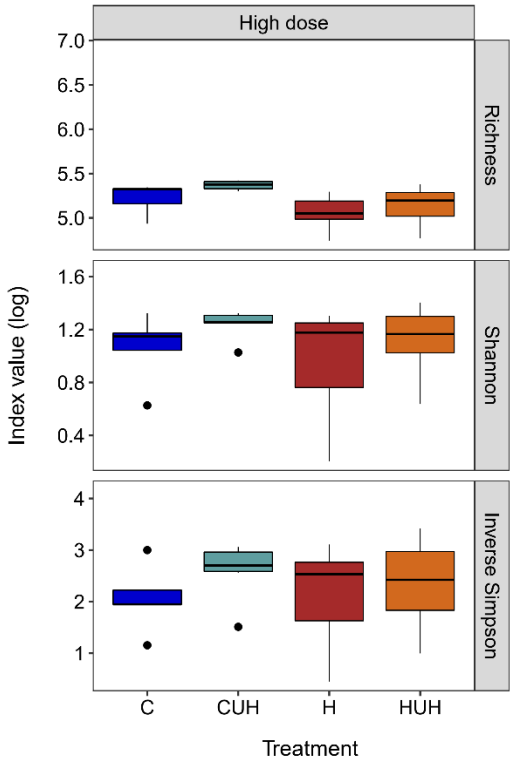


Figure 25: Effect of dietary supplementation with high dose of ulvan on alpha-diversity indexes of gut microbiota. Index values are presented as natural log. C, CS diet-fed rats; CUH, CS diet-fed rats supplemented with 2% ulvan; H, HCHF diet-fed rats; HUH, HCHF diet-fed rats supplemented with 2% ulvan.

Bacterial community structure and physiological response were correlated, and these are displayed in Table 17 (Mantel statistic r = .456; p = .001). The combination of the physiological variables 'Ulvan intake', 'Body weight gain %', 'Food intake 8-16 week' and 'Kidney weight' were found to have the maximum (rank) correlation with bacterial community structure (correlation = 0.75; function bioenv from vegan R package). A total of 21 OTUs were found to be strongly correlated with at least 6 of these 10 variables shown in Table 17 ($p < 0.05$, $R^2 > 0.5$). Of these, 14 OTUs belong to the phylum Firmicutes, 6 to the phylum Bacteroidetes and 1 to the phylum Proteobacteria (Supplementary Table 3). Both *Alistipes* and *Parabacteroides* were negatively correlated with features of obesity and positively correlated with ulvan intake (Table 18).

The normalised abundance of 10 OTUs that were strongly associated with several physiological variables are displayed in Figure 26. The abundance of OTU26 (bacterium from the phylum Firmicutes, family *Lachnospiraceae*) was negatively correlated to all physiological variables relating to fat mass and body weight but positively correlated to ulvan intake. This can be observed in Figure 26, as both CUH and HUH have a higher abundance than their respective diet controls, whereas the abundance of another OTU from the *Lachnospiraceae* family (OTU314) was negatively correlated to ulvan intake and positively correlated to body weight and fat mass. Bacteroidetes bacteria from the genera Alistipes (OTU130 and OTU59), Parabacteroides (OTU218 and OTU167) and Muribaculaceae (OTU232) were found at lower abundance in group H, compared to groups HUH, CUH and C.

Table 17: Correlation between gut bacterial community structure and physiological variables in high ulvan-treated rats.

Physiological variable	R^2	p-value
Liver weight	0.74	0.001
Food intake	0.67	0.001
Body weight	0.65	0.001
Kidney weight	0.63	0.001
Ulvan intake	0.63	0.001
Energy intake	0.61	0.001
Total fat mass	0.59	0.001
Retroperitoneal fat mass	0.54	0.002
Body weight gain %	0.52	0.001
Total abdominal fat pads	0.52	0.001

This table includes the physiological parameters with the strongest correlation with the microbial data ($R2 > 0.5$, $p < 0.05$; function envfit from vegan R package).

Table 18: OTUs correlated with six or more physiological parameters for high dose ulvan

OTU ID	Phylum	Family	Genus	Correlation with physiological parameters
OTU130	Bacteroidetes	Rikenellaceae	Alistipes	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Energy intake 8-16wk (-), Total fat mass (-), Kidney weight (-), Liver weight (-), Retroperitoneal fat mass (-), Ulvan intake (+)
OTU218	Bacteroidetes	Tannerellaceae	Parabacteroides	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Energy intake 8-16wk (-), Total fat mass (-), Kidney weight (-), Liver weight (-), Retroperitoneal fat mass (-), Ulvan intake (+)
OTU59	Bacteroidetes	Rikenellaceae	Alistipes	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Total fat mass (-), Food intake 8-16wk (+), Liver weight (-), Retroperitoneal fat mass (-), Ulvan intake (+)
OTU167	Bacteroidetes	Tannerellaceae	Parabacteroides	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Total fat mass (-), Liver weight (-), Retroperitoneal fat mass (-), Ulvan intake (+)

This table includes the physiological parameters that were found to be strongly correlated ($p < 0.05$, $R2 > 0.5$) with the microbial community ($n = 10$) and incorporates OTUs that interact with at least 6 of these parameters ($n = 21$). Plus sign (+) indicates positive interactions, while minus sign (-) indicates negative interactions.

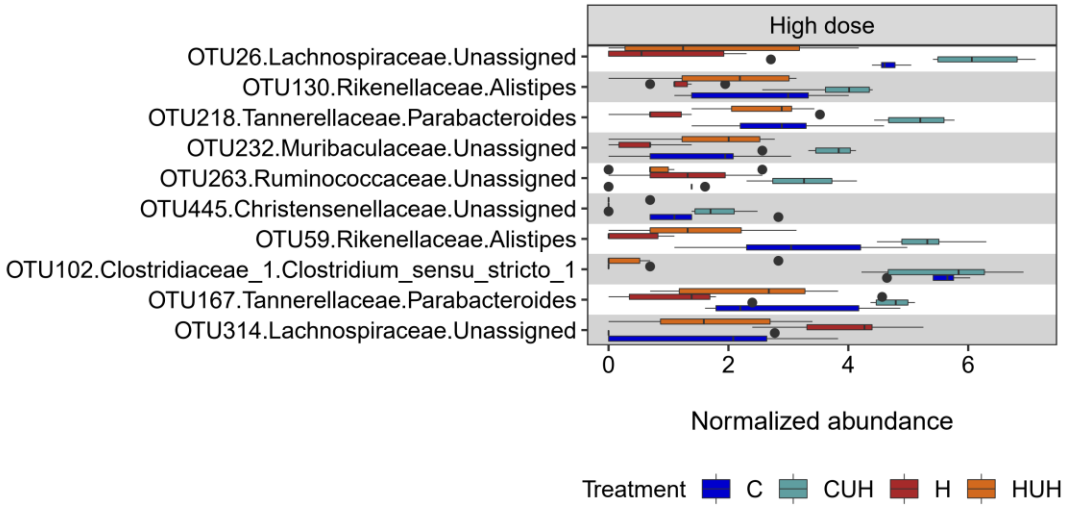


Figure 26: Response of the differentially abundant OTUs found to be strongly associated with several physiological variables. Normalised abundance was calculated by the R package mvabund. Plot shows OTU taxonomic classification at family and genus levels. C, CS diet-fed rats; CUH, CS diet-fed rats supplemented with 2% ulvan; H, HCHF diet-fed rats; HUH, HCHF diet-fed rats supplemented with 2% ulvan.

H rats had lower quantities of acetic acid and propionic acid in the caecum than CS diet-fed rats, although butyric acid was not changed by either diet or ulvan supplementation (Figure 27). The concentration of acetic acid in HUH rat caecum was intermediate to C and H rats, however there was no significant difference (Figure 27A).

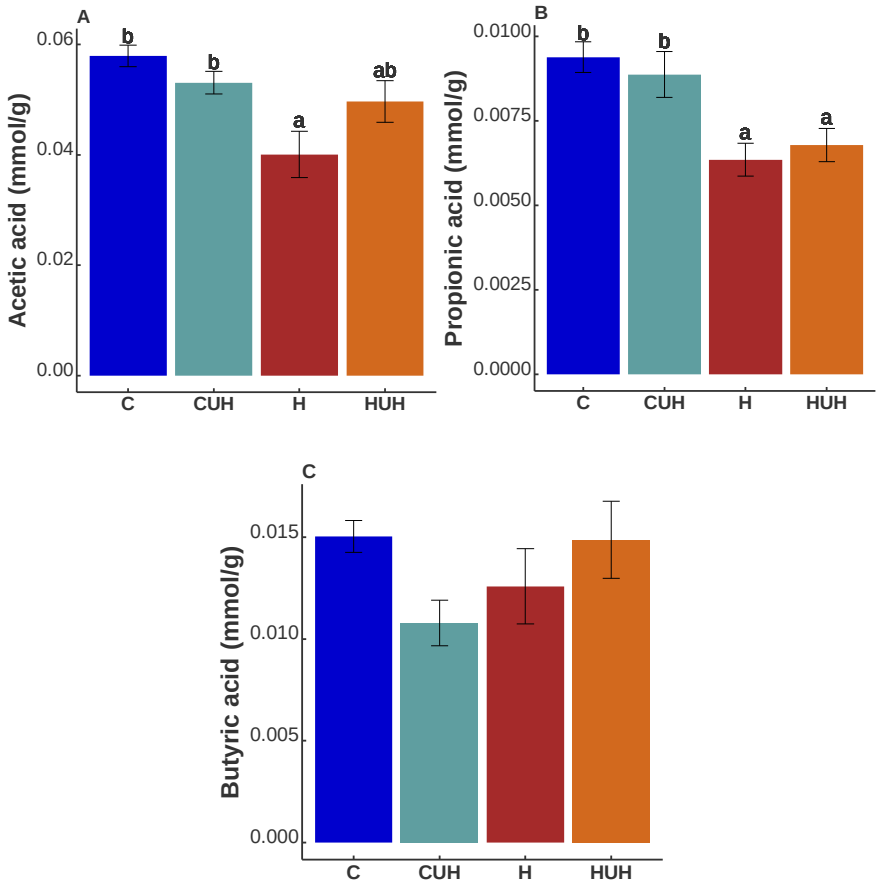


Figure 27: Caecal short chain fatty acid content. A Acetic acid mmol/g. B Propionic acid mmol/g. C Butyric acid mmol/g. Values are mean ± SEM, n = 6-8. Means without a common letter differ, p < 0.05. C, CS diet-fed rats; CUH, CS diet-fed rats supplemented with 2% ulvan; H, HCHF diet-fed rats; HUH, HCHF diet-fed rats supplemented with 2% ulvan.

3.3.3 Low dose ulvan

3.3.3.1 Body composition, diet intake and metabolic changes

Similar to the high dose study, low dose ulvan reduced body weight gain % and fat mass change % in HUL rats compared to H rats (Figure 28B and 28C). There was no difference between CUL and C rats for both body weight and fat mass (Figure 28A, 28B and 28C). Abdominal fat pad content was not reduced in HUL rats compared to H rats ($p = 0.085$, Figure 28D). Ulvan intake did not change food or water intake in CS or HCHF diet-fed groups (Table 19), however feed efficiency was reduced in HUL compared to H rats (Figure 28E). Similar to the high dose study, ulvan intake was unable to improve systolic

blood pressure, glucose tolerance, plasma triglycerides and total cholesterol (Table 19). Both plasma ALT and AST were reduced in CUL rats in comparison to C rats, and AST was reduced in HUL rats compared to H rats (Table 19).

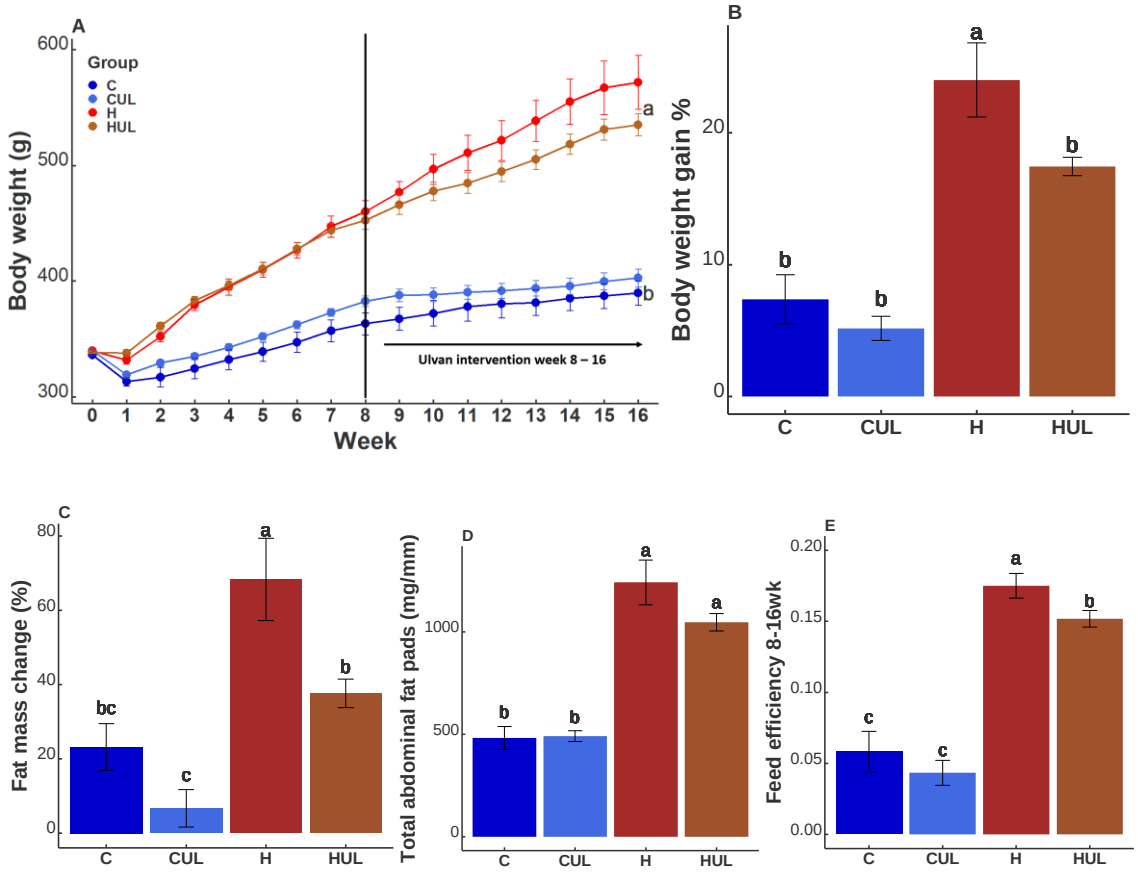


Figure 28: Body composition and diet intake. ***A*** *Body weight 0-16 weeks.* ***B*** *Change in fat mass % of body weight from 8-16 weeks.* ***C*** *Body weight gain % from 8-16 weeks.* ***D*** *Total abdominal fat pads mg tissue weight/mm tibial length. E Average daily feed conversion efficiency 8-16 weeks. Values are mean ± SEM, n = 6-12. Means without a common letter differ, p < 0.05. C, CS diet-fed rats; CUL, CS diet-fed rats supplemented with 0.5% ulvan; H, HCHF diet-fed rats; HUL, HCHF diet-fed rats supplemented with 0.5% ulvan.*

Table 19: Ulvan low dose physiological parameters

Variable	C	CUL	H	HUL	Two Way Anova		
					Diet	Treatment	Interaction
Ulvan intake (g/ kg body weight/day)	0 ± 0.0^{c}	0.49 ± 0.01^{a}	0 ± 0.0^{c}	0.23 ± 0.01^{b}	< 0.001	< 0.001	< 0.001
Body weight (g) week 8	363 ± 24^{b}	383 ± 16^{b}	460 ± 24^{a}	453 ± 27^{a}	< 0.001	0.462	0.104
Body weight (g) week 16	390 ± 11^{b}	403 ± 8^{b}	553 ± 17^{a}	535 ± 9^{a}	< 0.001	0.909	0.175
Water intake (g/day) week 0-8	35.4 ± 2.1	28.9 ± 2.0	31.6 ± 1.0	32.3 ± 0.8	0.5523	0.1153	0.0483
Water intake (g/day) week 8-16	27.9 ± 2.1^{ab}	25.2 ± 1.4^{b}	27.9 ± 0.7^{ab}	29.7 ± 0.6^{a}	0.0215	0.7649	0.1006
Food intake (g/day) week 0-8	42.4 ± 1.1^{a}	42.9 ± 0.6^{a}	25.0 ± 1.3^{b}	23.8 ± 0.6^{b}	< 0.001	0.709	0.329
Food intake (g/day) week 8-16	40.0 ± 1.4^{a}	39.5 ± 0.9^{a}	24.6 ± 1.3^{b}	23.0 ± 0.5^{b}	< 0.001	0.274	0.556
Energy intake (kJ/day) week 0-8	476 ± 13^{b}	482 ± 6^{b}	566 ± 20^{a}	549 ± 10^{a}	< 0.001	0.613	0.344
Energy intake (kJ/day) week 8-16	449 ± 16^{b}	444 ± 10^{b}	546 ± 23^{a}	524 ± 9^{a}	< 0.001	0.305	0.537
Abdominal circumference (cm) week 8	18.5 ± 0.4^{b}	19.0 ± 0.2^{b}	21.5 ± 0.3^{a}	21.5 ± 0.2^{a}	< 0.001	0.135	0.448
Abdominal circumference (cm) week 16	19.0 ± 0.4^{b}	19.5 ± 0.2^{b}	23.5 ± 0.6^{a}	23.0 ± 0.3^{a}	< 0.001	0.778	0.324
Lean mass (g) week 8	278 ± 9	280 ± 5	289 ± 11	273 ± 7	0.919	0.359	0.253
Lean mass (g) week 16	290 ± 9	296 ± 7	314 ± 12	297 ± 11	0.401	0.656	0.299
Fat mass (g) week 8	63 ± 13^{b}	88 ± 8^{b}	158 ± 15^{b}	171 ± 12^{b}	< 0.001	0.142	0.661
Fat mass (g) week 16	82 ± 5^{b}	91 ± 7^{b}	252 ± 17^{a}	224 ± 17^{a}	< 0.001	0.502	0.195
SBP (mmHg) week 16	127 ± 7	135 ± 4	145 ± 4	141 ± 3	0.021	0.679	0.153

Table 20: Ulvan low dose physiological parameters continued.

Variable	C	CUL	H	HUL	Two Way Anova		
					Diet	Treatment	Interaction
Tissue wet weight (mg/mm tibial length)							
Kidney	47.8 ± 1.8^{b}	48.6 ± 1.6^{b}	58.8 ± 2.2^{a}	56.5 ± 1.3^{a}	< 0.001	0.68	0.39
Omental fat	163 ± 11^{b}	174 ± 9^{b}	356 ± 24^{a}	323 ± 15^{a}	< 0.001	0.469	0.154
Epididymal fat	99 ± 24^{b}	119 ± 12^{b}	257 ± 37^{a}	204 ± 17^{a}	< 0.001	0.451	0.096
Retroperitoneal fat	220 ± 29^{b}	224 ± 17^{b}	628 ± 62^{a}	560 ± 32^{a}	< 0.001	0.363	0.308
Liver	234 ± 6^{b}	248 ± 8^{b}	381 ± 23^{a}	392 ± 17^{a}	< 0.001	0.437	0.923
Serum parameters							
Total cholesterol (mmol/L)	1.87 ± 0.08	1.75 ± 0.10	1.80 ± 0.13	1.64 ± 0.07	0.365	0.218	0.837
Triglycerides (mmol/L)	0.45 ± 0.05^{c}	0.61 ± 0.06^{bc}	1.21 ± 0.18^{a}	1.47 ± 0.21^{ab}	< 0.001	0.239	0.767
ALT (U/L)	27.6 ± 1.5^{a}	21.4 ± 1.0^{b}	29.8 ± 3.4^{a}	25.7 ± 0.8^{ab}	0.019	0.003	0.499
AST (U/L)	121.8 ± 7.3^{a}	75.7 ± 3.2^{b}	142.8 ± 18.1^{a}	72.3 ± 4.9^{b}	0.322	< 0.001	0.140
Oral glucose tolerance test							
AUC week 8 (mmol/L x min)	550 ± 25	549 ± 15	616 ± 26	611 ± 21	0.005	0.886	0.933
AUC week 16 (mmol/L x min)	532 ± 21	531 ± 11	611 ± 20	616 ± 20	< 0.001	0.911	0.870
Fasting blood glucose mmol/L week 16	2.6 ± 0.3^{b}	2.9 ± 0.1^{ab}	3.3 ± 0.2^{a}	3.3 ± 0.1^{a}	< 0.001	0.245	0.269

SBP, systolic blood pressure; ALT, alanine transaminase; AST, aspartate aminotransferase; AUC, area under the curve; C, CS diet-fed rats; CUL, CS diet-fed rats supplemented with 0.5% ulvan; H, HCHF diet-fed rats; HUL, HCHF diet-fed rats supplemented with 0.5% ulvan.

3.3.3.2 Liver changes

Low dose ulvan intake did not change liver weight (Table 19). There was no visible effect of low dose ulvan intake on liver fat deposition and inflammation (Figure 29).

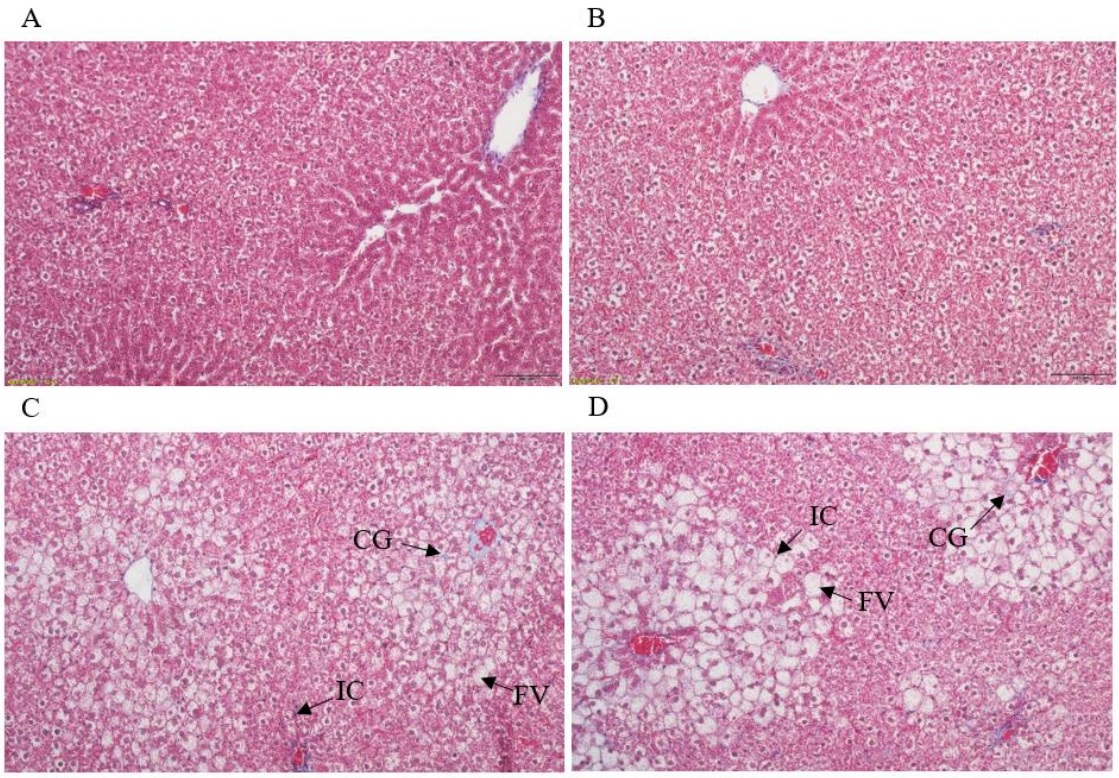


Figure 29: Hepatic structure. Masson's Trichrome staining of the liver showing fat deposition is marked as 'FV', inflammatory cells are marked as 'IC' and collagen is marked as 'CG', n = 4. Scale = 100 µm. ***A*** *C liver.* ***B*** *CUL liver.* ***C*** *H liver.* ***D*** *HUL liver. C, CS diet-fed rats; CUL, CS diet-fed rats supplemented with 0.5% ulvan; H, HCHF diet-fed rats; HUL, HCHF diet-fed rats supplemented with 0.5% ulvan. An enlarged image of HUL liver can be found in Supplementary Figure 1.*

3.3.3.3 Microbiome analysis

Distance-based multivariate analysis combining the abundances of all OTUs (n = 3706) showed that treatments have a distinct bacterial composition. Supplementation with a low dose of ulvan seems to have a more pronounced effect on animals fed a corn-starch-rich diet (C/CUL) than animals fed a high carbohydrate and high fat diet (H/HUL) (Figure 30A). PERMANOVA revealed that both diet ($p = 0.001$) and low ulvan supplementation ($p = 0.005$) have a statistically significant effect on the gut microbial community, while the interaction between diet and low ulvan did not produce a statistically significant effect ($p = 0.076$). Post-hoc pairwise comparisons demonstrated that, apart from H/HUL, all other pairs have distinct bacterial community composition ($0.004 < p < 0.0156$). The Bray-

Curtis dissimilarity matrices stress value was 0.105 for this non-metric multidimensional scaling plot.

Distance-based multivariate analyses combining data from 27 physiological parameters measured at week 16 showed that ordinate separation was more evident between groups that received different diets (C/CUH in relation to H/HUH) (Figure 30B). PERMANOVA revealed that both diet and supplement (but not their interaction) had a statistically significant effect on rat physiological performance (diet p = 0.001, ulvan p = 0.041, interaction p = 0.729). Post-hoc pairwise comparisons showed that the physiological responses between C and CUL (p = 0.277) and H and HUL (p = 0.124) were not statistically different. The Euclidean distance stress value was 0.03.

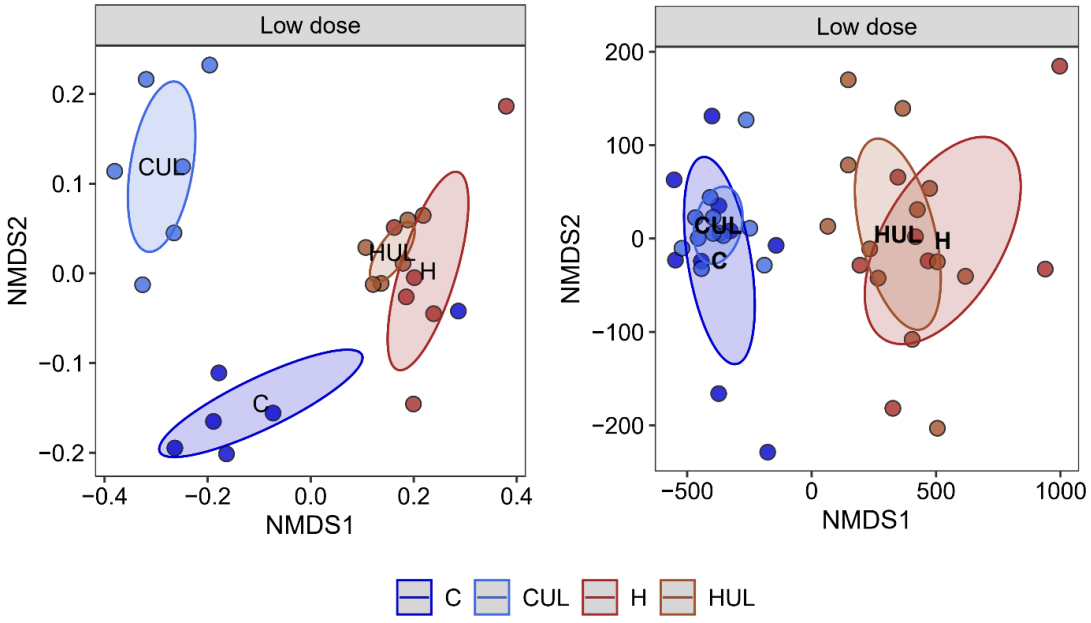


Figure 30: NMDS plots include data measured at week 16. In these plots, each point corresponds to one sample (n = 12 per treatment) and incorporates the response of 27 physiological parameters. ***A*** *Bacterial community structure. Non-metric multidimensional scaling (NMDS) plots incorporating all OTUs generated (n = 3706). In these plots, each point corresponds to one sample and incorporates the abundance of all OTUs present in that sample.* ***B*** *Physiological response of rats. C, CS diet-fed rats; CUL, CS diet-fed rats supplemented with 0.5% ulvan; H, HCHF diet-fed rats; HUL, HCHF diet-fed rats supplemented with 0.5% ulvan.*

Analysis of bacterial community structure across treatments showed that bacteria from the phylum Firmicutes were the most abundant, regardless of the diet or ulvan supplementation (Figure 31A). The relative abundance of bacteria from the phylum Bacteroidetes was affected by diet ($p = 0.0005$) and ulvan intake ($p = 0.0065$). In addition, the relative abundance of bacteria from the phylum Verrucomicrobia was reduced by the ulvan supplementation ($p = 0.0052$). This difference in bacterial composition was further shown by calculating the Firmicutes/Bacteroidetes (F/B) ratio, which was shown to be strongly impacted by diet ($p = 0.014$) (Figure 31B). Ulvan supplementation did not reduce the F/B ratio in the low dose study ($p = 0.086$), and only groups H and CUL were found to be different ($p = 0.022$). Low dose ulvan had an effect on Inverse Simpson diversity index (low dose: Inverse Simpson supplement $p = 0.018$) (Figure 32). Rats from the group HUL exhibited higher values for Inverse Simpson diversity than those of group C. In the low dose study, there was no effect of diet or ulvan on overall richness or Shannon diversity index.

Multivariate analysis of OTU distribution performed using the R package mvabund revealed that diet and supplement, as well as the interaction between diet and supplement, had a significant effect on bacterial community structure. In the low dose study, diet had a stronger effect on the bacterial community composition, affecting the abundance of 424 OTUs (11% of the gut microbiota). Supplementation with the low dose of ulvan altered the abundance of 139 OTUs (4%), while a total of 111 OTUs had its abundance affected by both diet and supplement (Supplementary Table 5).

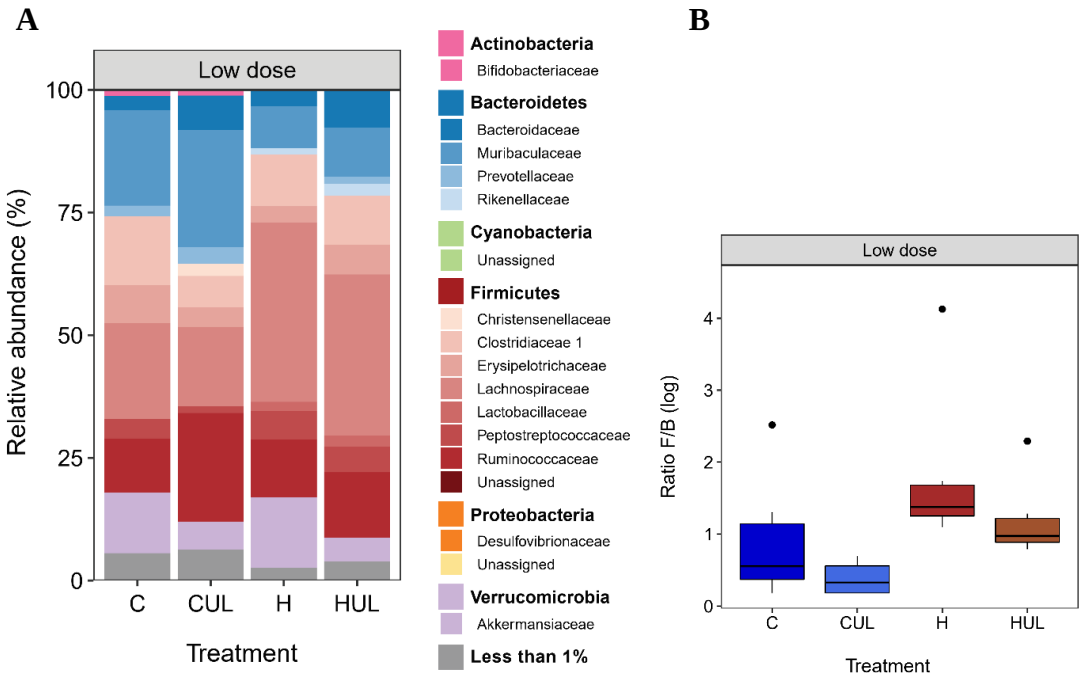


*Figure 31: Bacterial taxonomic composition of rat faecal samples. **A** Bacterial community at the phylum (in bold, main colours) and family levels, and include sequences with relative abundance higher than 1% (filtering performed at the family level). **B** Ratio of Firmicutes and Bacteroidetes abundances in the gut microbiota. C, CS diet-fed rats; CUL, CS diet-fed rats supplemented with 0.5% ulvan; H, HCHF diet-fed rats; HUL, HCHF diet-fed rats supplemented with 0.5% ulvan. Dots are outliers.*

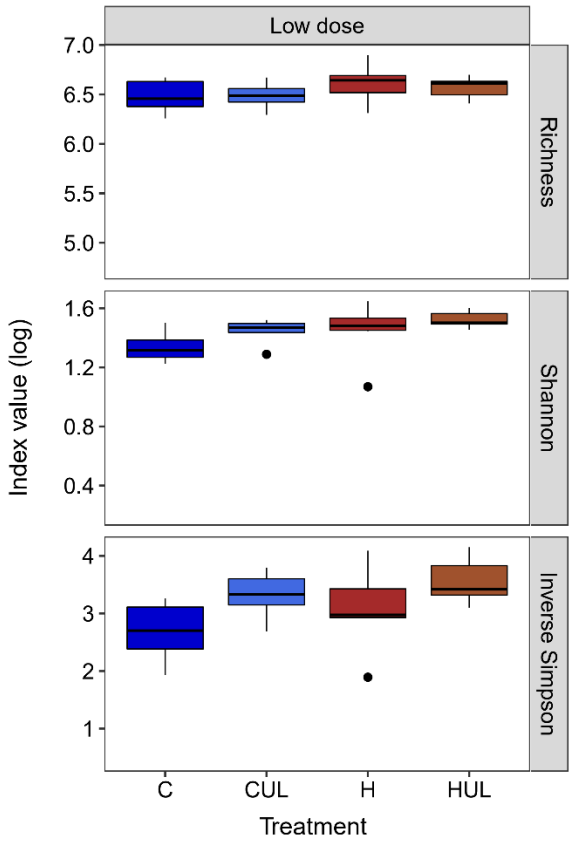


Figure 32: Effect of dietary supplementation with low dose of ulvan on alpha-diversity indexes of gut microbiota. Index values are presented as natural log. C, CS diet-fed rats; CUL, CS diet-fed rats supplemented with 0.5% ulvan; H, HCHF diet-fed rats; HUL, HCHF diet-fed rats supplemented with 0.5% ulvan. Dots are outliers

The analysis of the low dose data revealed that 58 OTUs were correlated with at least 6 of the 9 physiological variables included in this assessment ($p < 0.05$, $R2 > 0.5$). Of these, 49 OTUs belong to the phylum Firmicutes, 7 to the phylum Bacteroidetes, 1 to the phylum Proteobacteria and 1 to the phylum Actinobacteria (Supplementary Table 4). The combination of the variables ulvan intake, body weight gain %, food intake 8-16 weeks, water intake 8-16 weeks and omental fat mass were found to have the maximum (rank) correlation with bacterial community structure (correlation = 0.771; function bioenv from vegan R package). The abundance of OTU1154 (bacterium from the phylum Bacteroidetes, family *Muribaculaceae*) was negatively correlated to most of the physiological variables, apart from food intake 8-16 week and ulvan intake, which were

positively associated with the abundance of this sequence (Table 22) (R2 = 0.62 and 0.60, respectively). The correlation to ulvan intake is reflected by the increased abundance of OTU1154 in CUL rats (Figure 33). OTU1116 (phylum Firmicutes, family *Lachnospiraceae*, genus *Roseburia*) and OTU130 (phylum Firmicutes, family *Lachnospiraceae*) were correlated to 8 out of the 9 physiological parameters. The relative abundance of OTU1116 and OTU130 were higher in animals from groups H and HUL (relative to groups C and CUL) (Figure 33). Other bacteria from the family *Lachnospiraceae* (unassigned at the genus level), including OTU1696, OTU20 and OTU21, were also observed at higher abundance in groups H and HUL (Figure 33).

Table 21: Correlation between gut bacterial community structure and physiological variables.

Physiological variable	R^2	p-value
Ulvan intake	0.74	0.001
Omental fat mass fat mass	0.71	0.001
Food intake 8-16 weeks	0.70	0.001
Body weight gain %	0.65	0.001
Total abdominal fat pads	0.63	0.001
Body weight 16 week	0.58	0.001
Liver weight	0.55	0.001
Total fat mass	0.55	0.001
Retroperitoneal fat mass	0.53	0.001

This table includes the physiological parameters with the strongest correlation with the microbial data (R2 > 0.5, p < 0.05; function envfit from vegan R package).

Table 22: OTUs correlated with six or more physiological parameters for low dose ulvan

OTU ID	Phylum	Family	Genus	Correlation with physiological parameters
OTU103	Bacteroidetes	Tannerellaceae	Parabacteroides	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Food intake 8-16wk (+), Omental fat mass (-), Retroperitoneal fat mass (-)
OTU198	Bacteroidetes	Rikenellaceae	Alistipes	Total abdominal fat pads (-), Body weight gain % (-), Total fat mass (-), Food intake 8-16wk (+), Omental fat mass (-), Ulvan intake (+)
OTU1154	Bacteroidetes	Muribaculaceae	Unclassified	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Total fat mass (-), Food intake 8-16wk (+), Liver weight (-), Omental fat mass (-), Retroperitoneal fat mass (-), Ulvan intake (+)

This table includes the physiological parameters that were found to be strongly correlated ($p < 0.05$, $R2 > 0.5$) with the microbial community ($n = 10$) and incorporates OTUs that interact with at least 6 of these parameters ($n = 21$). Plus sign (+) indicates positive interactions, while minus sign (-) indicates negative interactions.

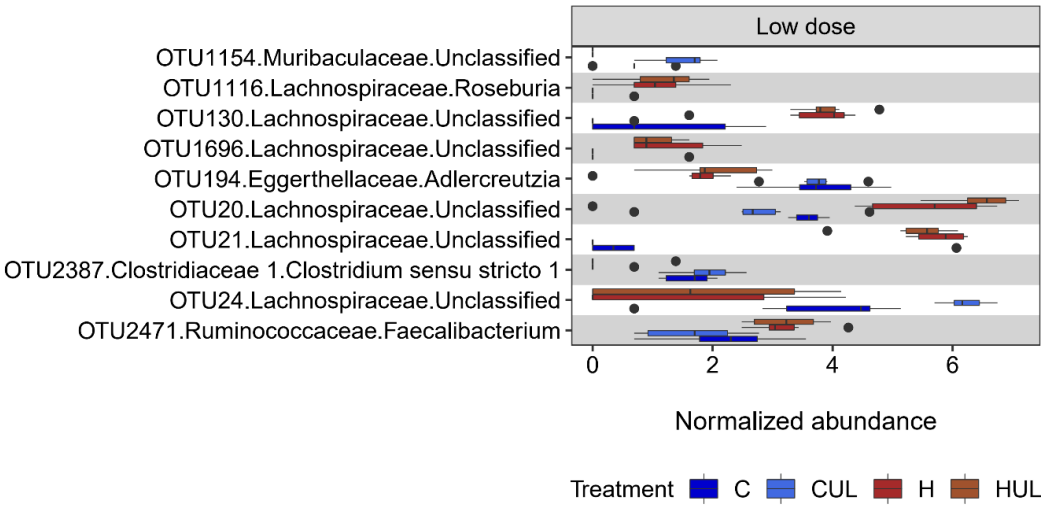


Figure 33: Response of the differentially abundant OTUs found to be strongly associated with several physiological variables. Normalised abundance was calculated by the R package mvabund. Plot shows OTU taxonomic classification at family and genus levels. C, CS diet-fed rats; CUL, CS diet-fed rats supplemented with 0.5% ulvan; H, HCHF diet-fed rats; HUL, HCHF diet-fed rats supplemented with 0.5% ulvan. Dots are outliers.

3.4 Discussion

In this study, the HCHF diet led to a phenotype associated with diet-induced metabolic syndrome including obesity, metabolic abnormalities, hypertension and ectopic fat deposition in the liver. This change was associated with a major shift in the gut microbiota profile, primarily a higher F/B ratio and decreased species diversity. Introducing ulvan into the diet reduced body weight gain, total fat mass gain and feed efficiency at both low (0.5%) and high (2%) doses. Additionally, the high dose of ulvan reduced deposition of fat in the liver and omental fat pad. The gut microbiota profile of the rats fed ulvan included major changes to species diversity and profile composition, and this has been correlated to the physiological changes observed. There were no changes to blood pressure or plasma lipids, which have been the major findings in previous ulvan and *Ulva* species studies (Hassan et al. 2011; Kumar et al. 2015; Ramirez-Higuera et al. 2014).

This is the first study to observe that purified ulvan reduces body weight gain and fat mass gain in obese rats. A comparable improvement was seen with both doses of ulvan tested, although further research is required to confirm that the dose-response curve to ulvan begins to taper off after 0.5%. This dose in the diet in HCHF diet-fed rats equated to 0.23 g/kg body weight/day. In a past study, a 0.5% diet inclusion of ulvan reduced the abdominal fat factor (abdominal fat weight/body weight × 100) but not body weight in a three week prevention study in rats (Pengzhan et al. 2003). A range of ulvan molecular weights were tested, from 28.2 kDa to 151.6 kDa, and this improvement was only observed with the lowest molecular weight tested (Pengzhan et al. 2003). In the current study, the average molecular weight of ulvan was 215.4 kDa with a polydispersity index of 3.7, suggesting that molecular weight may not be an important factor in inducing weight and fat loss as theorised previously. Differences in animal model used, in particular the shorter study length, may explain the absence of body weight change observed in the study by Pengzhan et al. (2003). Other previous studies have used the whole algal biomass and reported that 5% diet *Ulva ohnoi* reduced total body fat (Kumar et al. 2015) and 400 mg/kg body weight/day *Ulva linza* lowered abdominal fat in rats (Ramirez-Higuera et al. 2014). Both of these studies reported no change to body weight of *Ulva*-fed rats.

A dose of 250 mg/kg body weight/day in rats equates to approximately a 40 mg/kg body weight dose in humans, using the Reagan-Shaw calculation for rat-to-human scaling (Reagan-Shaw, Nihal & Ahmad 2008). Assuming an average human body weight of 60 kg, this is a daily fibre dose of 2.4 g for the low dose ulvan tested in this study and alternatively a dose of 9.7 g for the high dose ulvan tested.

Increased fibre intake is associated with decreased risk of cardiovascular disease and type 2 diabetes and reduced weight gain; however, this is dependent on the type of fibre consumed (Slavin 2013). High intake of fibre is not always beneficial, as dietary fibre increases faecal bulk and microbial gas production, and slows transit time, and an excess of these effects can lead to an increased risk of flatulence, constipation and the feeling of bloating (Bosaeus 2004). Further studies to understand how ulvan interacts with the digestive tract are necessary to determine risk of adverse effects from ulvan intake. Ulvan is a highly sulfated, water-soluble fibre that forms a gel in the presence of borate ions, calcium ions and a pH of 7.5 – 8, although it is not known whether ulvan forms a gel in the rat or human digestive tract (Lahaye & Robic 2007). Ulvan is resistant to both human endogenous enzyme digestion and bacterial fermentation (Andrieux et al. 1998; Bobin-Dubigeon et al. 1997; Durand et al. 1997). This supports the results of this study, as caecal short-chain fatty acid content was not changed by ulvan intake. In contrast, ulvan altered bacterial metabolism and caecal pH suggesting that ulvan is impacting the gut microbiota through an unknown mechanism (Andrieux et al. 1998). A small *in vitro* study (n = 5) found that 1% ulvan added to human faecal samples raised acetate concentrations at both the 12 hour and 24 hour check, although propionate and butyrate concentrations remained unchanged (Seong et al. 2019). This is similar to the results seen in the high dose ulvan tested in this study, as acetic acid was approaching significant difference to the HCHF control and perhaps a higher dose as used by Seong et al. (2019) would have led to this result.

This is also the first study to examine the effects of ulvan or the whole Ulva algal biomass on the gut microbiome *in vivo*. Ulvan supplementation (both low and high doses) produced pronounced changes to the gut microbiome composition. The F/B ratio has been extensively used as a biomarker for obesity in humans and other mammals (Koliada et al.

2017). There have been numerous studies confirming that this occurrence is more than an effect of diet or obesity itself, as F/B ratio is increased in obese people compared to lean people, and such a difference tends to decrease with weight loss (Clemente et al. 2012; Ley et al. 2006; Ridaura et al. 2013). In this study, we found that ulvan supplementation decreased the F/B ratio, independent of whether the rats were fed a CS or a HCHF diet. This was particularly pronounced in the high dose HUH rats, whose F/B ratio (10.9) was markedly reduced when compared to the H rats F/B ratio of 64. This reduction in the F/B ratio suggests that ulvan may potentially be producing the reduction to body weight gain and fat mass through modification of the gut microbiota.

To further examine this possibility, the physiological variables and gut microbiota were analysed for positive and negative correlations with the aim of discovering genera that have important impacts on physiology. A portion of OTUs (high dose = 21, low dose = 58) correlated with more than six physiological variables. In both studies, the genus *Alistipes* was increased in response to ulvan intake and was also correlated with reduced abdominal fat, body weight, and total fat mass. Increased abundance of *Alistipes* and also another genus *Prevotella* are considered to have a protective role in preventing non-alcoholic fatty liver disease (Jiang et al. 2015). *Prevotella* abundance was markedly increased in ulvan-supplemented rats in the high dose study (Figure 24), and this combined with *Alistipes* correlation to reduced liver weight (high dose only) suggests that these genera have potentially had a role in the reduced ectopic fat in the liver of HUH rats compared to H rats. *Alistipes* was more abundant in patients successful in losing and maintaining weight loss (Louis et al. 2016).

Parabacteroides spp. were negatively correlated with increased body weight and fat mass in both studies and were also positively correlated with ulvan intake in the high dose study (Supplementary Table 3 and

Supplementary Table 4). This genus was negatively correlated with inflammatory bowel disease (Walters, Xu & Knight 2014), but also in contrast to the current study has previously been linked to an increase in body weight (Lecomte et al. 2015; Wang et al. 2016). Two species from this genus have recently been identified to produce anti-obesity effects. *Parabacteroides goldsteinii* was elevated in response to high molecular weight

fungal polysaccharides, and further testing showed that it directly reduced obesity and maintained the gut barrier integrity (Wu et al. 2019). *Parabacteroides distasonis* transformed primary bile acids into secondary bile acids, and subsequently suppressed hepatic lipogenesis and increased hepatic fatty acid oxidation (Wang et al. 2019), ultimately reducing body weight gain from diet-induced obesity. A limitation of the current study was that the gut microbiota analysis could not confidently identify bacteria to the species level. Determining which *Parabacteroides* species is elevated in abundance by ulvan supplementation is an important step in identifying the mechanism behind body weight and fat mass reduction.

Ulvan supplementation (both low and high) reduced the relative abundance of *Akkermansia* spp (Figure 24 and Figure 31). There are only two known species within the Akkermansia genus, *Akkermansia muciniphila* and *Akkermansia glycaniphila*, and both are mucin-degrading bacteria (Ouwerkerk et al. 2016; Xing et al. 2019). This genus has anti-inflammatory effects through regulating the thickness of the gut mucus and maintaining gut barrier integrity to prevent lipopolysaccharides (LPS) uptake into serum (Zhou 2017). Increased serum LPS is associated with inflammatory bowel disease, type 2 diabetes mellitus, non-alcoholic fatty liver disease and other metabolic diseases (Cani et al. 2009; DeGruttola et al. 2016). Therefore, there is the potential for long-term effects due to the reduction of *Akkermansia* spp. as has been observed with high doses of carrageenan, another highly sulphated, soluble seaweed polysaccharide (Shang, Sun, et al. 2017). The mechanisms behind this ulvan-induced reduction of *Akkermansia* and possible subsequent effects (such as elevated serum LPS) are an important area for future study. Determining the *Akkermansia* species affected is also important, as *A. glycaniphila* does not contain the outer-membrane protein (Amuc_1100) that is related to the *Akkermansia*-induced changes to fat mass and metabolic syndrome (Ouwerkerk et al. 2016).

This model of diet-induced metabolic syndrome in rats has been used previously to test *Kappaphycus alvarezii* and *Sarconema filiforme*, which are two other seaweed species rich in another soluble fibre known as carrageenan (Du Preez et al. 2020; Wanyonyi et al. 2017). In these studies, the whole seaweed (powder) was provided in the food at a dose of 5% total diet. This dose of the whole algae equated to approximately 1.7% soluble fibre

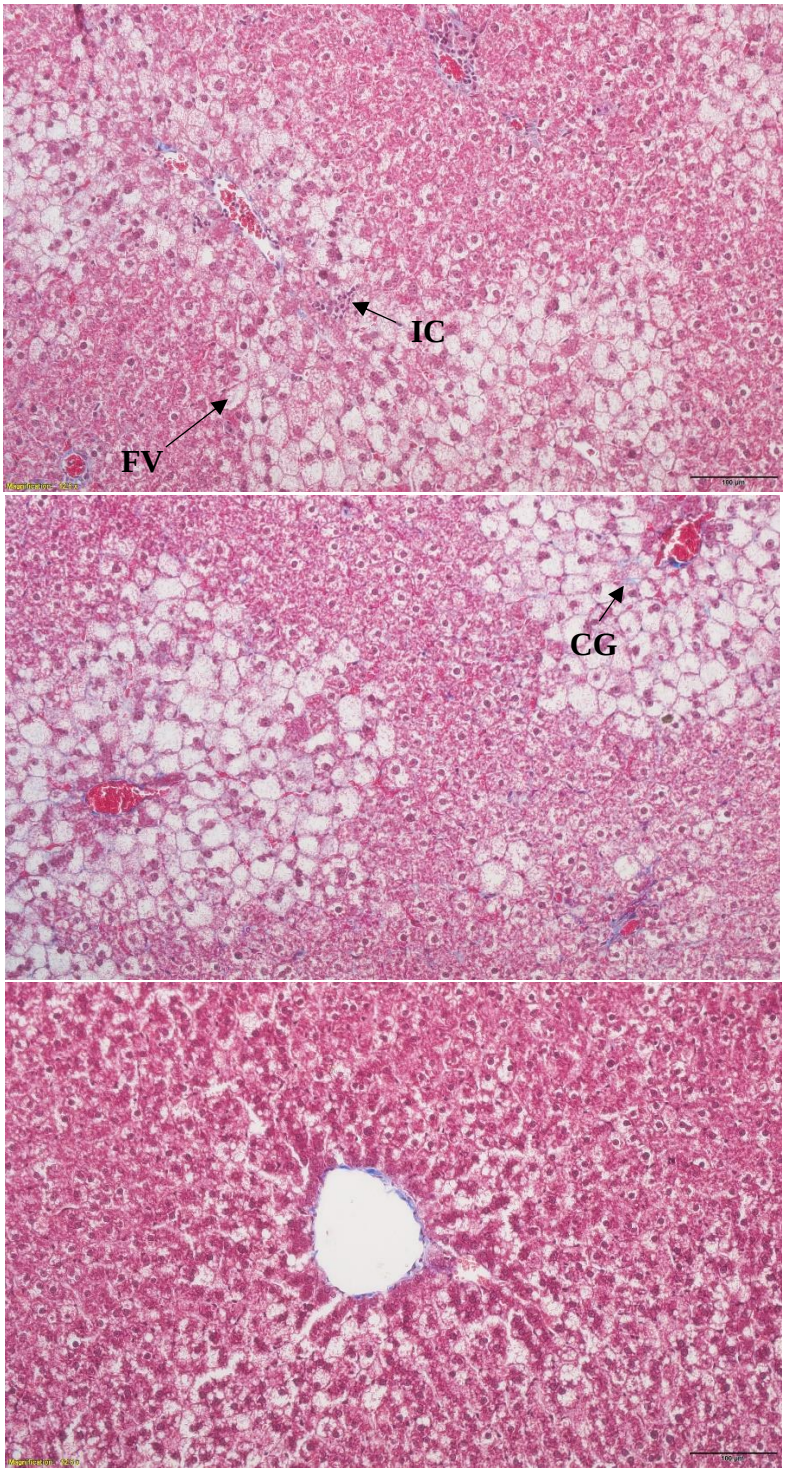
and 0.6% for *K. alvarezii* and *S. filiforme* respectively, as well as containing additional insoluble fibre, protein and minerals (Du Preez et al. 2020; Wanyonyi et al. 2017). In comparison to the current ulvan study, the anti-obesity effect of these seaweeds was considerably higher and additionally systolic blood pressure and plasma lipids were lower than controls. It is unclear whether this was due to differences between carrageenan and ulvan, or whether the other components of the whole algae led to this greater physiological response to *K. alvarezii* and *S. filiforme* over ulvan extracted from *U. ohnoi*. In the previous study testing *U. ohnoi* in this model of diet-induced metabolic syndrome, there were reductions to systolic blood pressure and improved glucose tolerance that were not observed in this current study (Kumar et al. 2015). This suggests that another component within the seaweed is responsible for the reduced systolic blood pressure, perhaps peptides with angiotensin-converting enzyme inhibiting function that have been reportedly found in other species of *Ulva* (Sun et al. 2019*)*. Further understanding of how to isolate and identify of these peptides within *Ulva* would allow possible combinations of both ulvan and these blood pressure-reducing peptides.

A consistent finding in previous ulvan studies is a reduction in serum plasma and triglycerides, and this was not the case in this current study (Qi, H et al. 2012; Qi & Sheng 2015; Ramirez-Higuera et al. 2014). Previous studies used a prevention model design, whereby ulvan was supplemented into the high-fat/high-carbohydrate diet from the start of the study. Ulvan may not be able to reverse high serum triglycerides and cholesterol concentrations and is more suited as a preventative intervention for this purpose. Further work to determine the mechanism by which ulvan reduced body weight and fat mass is necessary to ascertain whether ulvan can be used as a nutraceutical or functional food in obese humans. This would guide selection of patients who would most benefit from ulvan intake, perhaps through initial testing to analyse their gut microbiome to determine whether they would be an ideal candidate for the ulvan-induced changes observed previously in this study. Another consideration before ulvan can be considered a nutraceutical or functional food is to ensure that it is safe for human consumption. The analysis of ulvan composition in this study found that the detected quantities of arsenic, cadmium, mercury and lead were below the tolerable limits advised by Food Standards Australia (FSANZ 2003). This suggests that ulvan is safe for human consumption.

However, further testing in humans is necessary and due to the variable nature of seaweed content, ulvan should be reanalysed for each study.

In conclusion, ulvan is a promising soluble fibre due to the anti-obesity effects shown in this study and the well-recognised rapid growth of *Ulva* both in aquaculture and naturally forming in green tides. Despite this and the worldwide distribution of the seaweed, *Ulva* is still poorly utilised as there have only been limited cases of *Ulva* cultivation reported such as in abalone farms in South Africa (Bolton et al. 2009). Other tropical seaweeds such as *Caulerpa* and *Sargassum* are widely grown in developing countries such as the Philippines, Indonesia and Vietnam, and this is due to the easy accessibility of seaweed collection and cultivation (John et al. 2020). Further research to determine the health benefits of ulvan and to discover other uses of *Ulva,* such as high lipid and protein yield in comparison to other traditional crops (Ainsworth et al. 2012; Grieshop & Fahey 2001; Mata et al. 2016), would encourage the cultivation and commercial production of *Ulva* products in Australia and other developing countries, thus fully utilising a seaweed that is sustainable and native to the Australian coastline.

3.5 Supplementary



Supplementary Figure 1: Masson's staining of the liver to show fat deposition and collagen. Fat vacuoles marked as 'FV', inflammatory cells are marked as 'IC' and collagen is marked as 'CG', n = 4. Scale = 100 µm. ***A*** *H.* ***B*** *HUL.* ***C*** *HUH.*

Supplementary Table 1: Summary of statistical tests on differential OTU abundance in high-dose ulvan study

Global test (GLM) by mvabund		
Test statistic = 64.1 Global p-value = 0.001	Diet p-value = 0.001 Supplement p-value = 0.001 Diet * supplement p-value = 0.012	
Univariate analysis by mvabund (p < 0.05)		
Factor	Number of differentially abundant OTUs	% of total number of OTUs
Diet	130	37%
Supplement (high dose)	78	22%
Both diet and supplement	28	8%
Diet * supplement	43	12%
Total (unique OTUs)	198	57%

Bacterial abundance data were analysed using negative binomial multivariate generalised linear models (R package mvabund). ANOVA function with resampling-based univariate p-values in mvabund was used to identify the OTUs whose abundance was affected by diet, supplement and/or their interaction

Supplementary Table 2: Summary of statistical tests on differential OTU abundance from low dose ulvan study.

Global test (GLM) by mvabund	
Test statistic = 93.52	Diet p-value = 0.002
Global p-value = 0.001	Supplement p-value = 0.006
	Diet * supplement p-value = 0.003

Univariate analysis by mvabund (p < 0.05)		
Factor	Number of differentially abundant OTUs	% of total number of OTUs
Diet	424	11%
Supplement (low dose)	139	4%
Both diet and supplement	63	2%
Diet * supplement	111	3%
Total (unique OTUs)	776	21%

Bacterial abundance data were analysed using negative binomial multivariate generalised linear models (R package mvabund). ANOVA function with resampling-based univariate p-values in mvabund was used to identify the OTUs whose abundance was affected by diet, supplement and/or their interaction.

Supplementary Table 3: OTUs correlated with six or more physiological parameters for high dose ulvan.

OTU ID	Phylum	Family	Genus	Correlation with physiological parameters
OTU26	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Energy intake 8-16wk (-), Total fat mass (-), Food intake 8-16wk (+), Kidney weight (-), Liver weight (-), Retroperitoneal fat mass (-), Ulvan intake (+)
OTU130	Bacteroidetes	Rikenellaceae	Alistipes	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Energy intake 8-16wk (-), Total fat mass (-), Kidney weight (-), Liver weight (-), Retroperitoneal fat mass (-), Ulvan intake (+)
OTU218	Bacteroidetes	Tannerellaceae	Parabacteroides	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Energy intake 8-16wk (-), Total fat mass (-), Kidney weight (-), Liver weight (-), Retroperitoneal fat mass (-), Ulvan intake (+)
OTU232	Bacteroidetes	Muribaculaceae	Unclassified	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Energy intake 8-16wk (-), Kidney weight (-), Liver weight (-), Retroperitoneal fat mass (-), Ulvan intake (+)
OTU263	Firmicutes	Ruminococcaceae	Unclassified	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Energy intake 8-16wk (-), Kidney weight (-), Liver weight (-), Retroperitoneal fat mass (-), Ulvan intake (+)
OTU445	Firmicutes	Christensenellaceae	Unclassified	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Total fat mass (-), Food intake 8-16wk (+), Kidney weight (-), Liver weight (-), Retroperitoneal fat mass (-)
OTU59	Bacteroidetes	Rikenellaceae	Alistipes	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Total fat mass (-), Food intake 8-16wk (+), Liver weight (-), Retroperitoneal fat mass (-), Ulvan intake (+)
OTU102	Firmicutes	Clostridiaceae_1	Clostridium_sensu_stricto_1	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Food intake 8-16wk (+), Kidney weight (-), Liver weight (-), Retroperitoneal fat mass (-)
OTU167	Bacteroidetes	Tannerellaceae	Parabacteroides	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Total fat mass (-), Liver weight (-), Retroperitoneal fat mass (-), Ulvan intake (+)
OTU314	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Energy intake 8-16wk (+), Total fat mass (+), Retroperitoneal fat mass (+), Ulvan intake (-)
OTU514	Bacteroidetes	Muribaculaceae	Unclassified	Total abdominal fat pads (-), Body weight 16wk (-), Total fat mass (-), Food intake 8-16wk (+), Kidney weight (-), Liver weight (-), Retroperitoneal fat mass (-)

OTU558	Firmicutes	Lachnospiraceae	Roseburia	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Energy intake 8-16wk (+), Total fat mass (+), Kidney weight (+), Retroperitoneal fat mass (+)
OTU137	Firmicutes	Ruminococcaceae	Oscillibacter	Total abdominal fat pads (+), Body weight 16wk (+), Total fat mass (+), Food intake 8-16wk (-), Liver weight (+), Retroperitoneal fat mass (+)
OTU18	Firmicutes	Ruminococcaceae	Unclassified	Body weight 16wk (-), Body weight gain % (-), Energy intake 8-16wk (-), Food intake 8-16wk (+), Kidney weight (-), Liver weight (-)
OTU227	Firmicutes	Lachnospiraceae	Lachnoclostridium	Total abdominal fat pads (+), Body weight 16wk (+), Energy intake 8-16wk (+), Total fat mass (+), Liver weight (+), Retroperitoneal fat mass (+)
OTU240	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Total fat mass (+), Food intake 8-16wk (-), Liver weight (+), Retroperitoneal fat mass (+)
OTU354	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Total fat mass (+), Food intake 8-16wk (-), Liver weight (+), Retroperitoneal fat mass (+)
OTU387	Firmicutes	Streptococcaceae	Streptococcus	Body weight 16wk (-), Total fat mass (-), Food intake 8-16wk (+), Kidney weight (-), Liver weight (-), Ulvan intake (+)
OTU44	Firmicutes	Ruminococcaceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Total fat mass (+), Food intake 8-16wk (-), Liver weight (+), Retroperitoneal fat mass (+)
OTU5	Firmicutes	Clostridiales_vadinBB60_group	Unclassified	Body weight 16wk (-), Energy intake 8-16wk (-), Kidney weight (-), Liver weight (-), Retroperitoneal fat mass (-), Ulvan intake (+)
OTU80	Proteobacteria	Desulfovibrionaceae	Desulfovibrio	Total abdominal fat pads (-), Energy intake 8-16wk (-), Kidney weight (-), Liver weight (-), Retroperitoneal fat mass (-), Ulvan intake (+)

Supplementary Table 4: OTUs correlated with six or more physiological parameters for low dose ulvan

OTU ID	Phylum	Family	Genus	Correlation with physiological parameters
OTU1154	Bacteroidetes	Muribaculaceae	Unclassified	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Total fat mass (-), Food intake 8-16wk (+), Liver weight (-), Omental fat mass (-), Retroperitoneal fat mass (-), Ulvan intake (+)
OTU1116	Firmicutes	Lachnospiraceae	Roseburia	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Food intake 8-16wk (-), Liver weight (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU130	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Food intake 8-16wk (-), Liver weight (+), Omental fat mass (+), Retroperitoneal fat mass (+)

OTU1696	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Food intake 8-16wk (-), Liver weight (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU194	Actinobacteria	Eggerthellaceae	Adlercreutzia	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Total fat mass (-), Food intake 8-16wk (+), Liver weight (-), Omental fat mass (-), Retroperitoneal fat mass (-)
OTU20	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Food intake 8-16wk (-), Liver weight (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU21	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Food intake 8-16wk (-), Liver weight (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU2387	Firmicutes	Clostridiaceae 1	Clostridium sensu stricto 1	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Total fat mass (-), Food intake 8-16wk (+), Liver weight (-), Omental fat mass (-), Retroperitoneal fat mass (-)
OTU24	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Total fat mass (-), Food intake 8-16wk (+), Omental fat mass (-), Retroperitoneal fat mass (-), Ulvan intake (+)
OTU2471	Firmicutes	Ruminococcaceae	Faecalibacterium	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Food intake 8-16wk (-), Liver weight (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU32	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Food intake 8-16wk (-), Liver weight (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU33	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Food intake 8-16wk (-), Liver weight (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU358	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Food intake 8-16wk (-), Liver weight (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU37	Bacteroidetes	Muribaculaceae	Unclassified	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Total fat mass (-), Food intake 8-16wk (+), Liver weight (-), Omental fat mass (-), Retroperitoneal fat mass (-)
OTU436	Proteobacteria	Desulfovibrionaceae	Bilophila	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Total fat mass (-), Food intake 8-16wk (+), Liver weight (-), Omental fat mass (-), Retroperitoneal fat mass (-)

OTU440	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Food intake 8-16wk (-), Liver weight (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU567	Firmicutes	Ruminococcaceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Food intake 8-16wk (-), Liver weight (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU63	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Food intake 8-16wk (-), Liver weight (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU85	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Food intake 8-16wk (-), Liver weight (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU874	Firmicutes	Clostridiaceae 1	Clostridium sensu stricto 1	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Total fat mass (-), Food intake 8-16wk (+), Liver weight (-), Omental fat mass (-), Retroperitoneal fat mass (-)
OTU987	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Food intake 8-16wk (-), Liver weight (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU1280	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Liver weight (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU1960	Firmicutes	Lachnospiraceae	Acetatifactor	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Liver weight (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU2369	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Liver weight (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU2468	Firmicutes	Lachnospiraceae	Acetatifactor	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Liver weight (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU2474	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Liver weight (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU255	Firmicutes	Christensenellaceae	Unclassified	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Food intake 8-16wk (+), Omental fat mass (-), Retroperitoneal fat mass (-), Ulvan intake (+)

OTU2762	Firmicutes	Clostridiaceae 1	Clostridium sensu stricto 1	Body weight 16wk (-), Body weight gain % (-), Total fat mass (-), Food intake 8-16wk (+), Liver weight (-), Omental fat mass (-), Retroperitoneal fat mass (-)
OTU2903	Firmicutes	Ruminococcaceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Total fat mass (+), Food intake 8-16wk (-), Liver weight (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU317	Firmicutes	Family XIII	Unclassified	Total abdominal fat pads (-), Body weight 16wk (-), Total fat mass (-), Food intake 8-16wk (+), Liver weight (-), Omental fat mass (-), Retroperitoneal fat mass (-)
OTU3372	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Liver weight (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU3511	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Food intake 8-16wk (-), Liver weight (+), Omental fat mass (+)
OTU40	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Liver weight (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU55	Bacteroidetes	Muribaculaceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Food intake 8-16wk (-), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU677	Firmicutes	Lachnospiraceae	Roseburia	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Liver weight (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU721	Firmicutes	Lachnospiraceae	Lachnoclostridium	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Liver weight (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU8	Bacteroidetes	Muribaculaceae	Unclassified	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Total fat mass (-), Food intake 8-16wk (+), Retroperitoneal fat mass (-), Ulvan intake (+)
OTU835	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Food intake 8-16wk (-), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU877	Firmicutes	Ruminococcaceae	Oscillibacter	Body weight 16wk (-), Body weight gain % (-), Total fat mass (-), Food intake 8-16wk (+), Omental fat mass (-), Retroperitoneal fat mass (-), Ulvan intake (+)

OTU9	Bacteroidetes	Bacteroidaceae	Bacteroides	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Food intake 8-16wk (+), Omental fat mass (-), Retroperitoneal fat mass (-), Ulvan intake (+)
OTU103	Bacteroidetes	Tannerellaceae	Parabacteroides	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Food intake 8-16wk (+), Omental fat mass (-), Retroperitoneal fat mass (-)
OTU140	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Food intake 8-16wk (-), Omental fat mass (+)
OTU1458	Firmicutes	Ruminococcaceae	Ruminiclostridium 9	Total abdominal fat pads (-), Body weight gain % (-), Total fat mass (-), Food intake 8-16wk (+), Omental fat mass (-), Retroperitoneal fat mass (-)
OTU166	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Total fat mass (+), Food intake 8-16wk (-), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU186	Firmicutes	Ruminococcaceae	Oscillibacter	Total abdominal fat pads (-), Body weight 16wk (-), Body weight gain % (-), Total fat mass (-), Omental fat mass (-), Retroperitoneal fat mass (-)
OTU1934	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU198	Bacteroidetes	Rikenellaceae	Alistipes	Total abdominal fat pads (-), Body weight gain % (-), Total fat mass (-), Food intake 8-16wk (+), Omental fat mass (-), Ulvan intake (+)
OTU2208	Firmicutes	Ruminococcaceae	Ruminiclostridium 9	Total abdominal fat pads (-), Body weight 16wk (-), Total fat mass (-), Food intake 8-16wk (+), Omental fat mass (-), Retroperitoneal fat mass (-)
OTU231	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Food intake 8-16wk (-), Omental fat mass (+)
OTU2551	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Total fat mass (+), Food intake 8-16wk (-), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU2646	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU2953	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Liver weight (+), Omental fat mass (+)

OTU3041	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU3120	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU333	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Total fat mass (+), Food intake 8-16wk (-), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU3463	Firmicutes	Ruminococcaceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Food intake 8-16wk (-), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU61	Firmicutes	Lachnospiraceae	Lachnoclostridium	Total abdominal fat pads (+), Body weight 16wk (+), Total fat mass (+), Food intake 8-16wk (-), Omental fat mass (+), Retroperitoneal fat mass (+)
OTU985	Firmicutes	Lachnospiraceae	Unclassified	Total abdominal fat pads (+), Body weight 16wk (+), Body weight gain % (+), Total fat mass (+), Liver weight (+), Omental fat mass (+)

This table includes the physiological parameters that were found to be strongly correlated ($p < 0.05$, $R2 > 0.5$) with the microbial community ($n = 10$) and incorporates OTUs that interact with at least 6 of these parameters ($n = 58$). Plus sign (+) indicates positive interactions, while minus sign (-) indicates negative interaction

CHAPTER 4 – EFFECTS OF FUCOXANTHIN ON DIET-INDUCED METABOLIC SYNDROME IN RATS

4.1 Introduction

Fucoxanthin is a yellow-brown carotenoid in brown macroalgae and diatoms that forms part of a light-harvesting complex with chlorophyll necessary for photosynthesis (Owens 1986). Diatoms are unicellular, eukaryotic organisms that have relatively high nutrient uptake rates compared to other microalgae (Litchman 2007). For example, the diatom *Phaeodactylum tricornutum* contains on average 36% crude protein, 26% carbohydrates, 18% lipids and 16% ash, as well as over 1500 mg of calcium and potassium per 100 g dry biomass (Rebolloso-Fuentes et al. 2001). *P. tricornutum* has a yield of 16.33 mg/g dry weight fucoxanthin, which is much higher than the yield from commonly consumed brown macroalgae such as *Eisenia bicyclis* (1.3 mg/g dry weight) and *Undaria pinnatifida* (0.98 mg/g dry weight) (Grasa-López et al. 2016; Kim et al. 2012). Further, diatoms are capable of doubling their biomass in a few hours (Wang & Seibert 2017) and are highly responsive to changes in their environmental conditions, allowing manipulation of their nutrient levels through modulating factors such as light intensity, nitrogen and temperature (Kim et al. 2012; Pasquet et al. 2014). For these reasons, diatoms such as *P. tricornutum* and *Odentella aurita* are commercially promising, particularly as the diatom can potentially be utilised for multiple revenue streams due to the variety of nutrients in the biomass (Wang & Seibert 2017).

Carotenoids, including fucoxanthin, are well-acknowledged for their antioxidant properties due to the large number of conjugated double bonds within the hydrocarbon backbone allowing for potent free radical scavenging and singlet oxygen quenching activities (Figure 34) (Dutta, Chaudhuri & Chakraborty 2005). Fucoxanthin's unusual structural features of an allenic bond (one carbon atom has two double bonds) and an epoxide group (oxygen atom attached to two adjacent carbon atoms) is thought to further contribute to the anti-obesity, anti-cancer and anti-diabetic effects shown in rodents and *in vitro* models (Bae et al. 2020). Much of this previous research has been focused on the anti-obesity potential of fucoxanthin (Grasa-López et al. 2016;

Hosokawa et al. 2010; Maeda et al. 2015; Woo et al. 2009). The results from rodent studies in short, prevention models have shown that fucoxanthin can reduce body weight and white adipose tissue through increasing lipolysis and UCP-1 expression and decreasing lipogenesis and inflammation in adipose tissue (Grasa-López et al. 2016; Hosokawa et al. 2010; Woo et al. 2009).

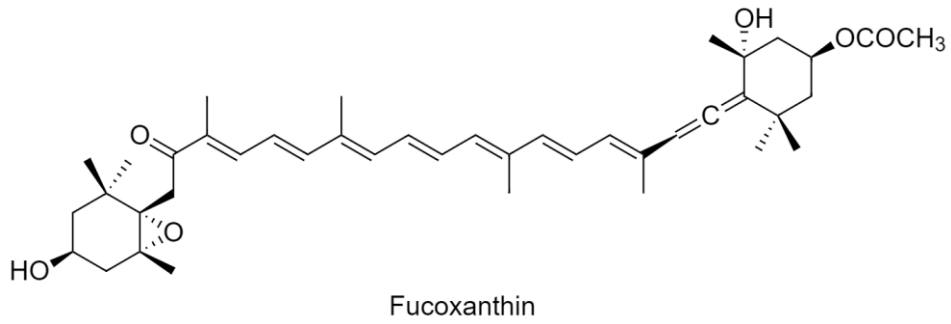


Figure 34: Structure of fucoxanthin

These anti-obesity effects are potentially due to the preference for fucoxanthin storage in adipose tissue. A study in obese/diabetic mice (Airanthi et al. 2011) has shown that approximately 80% of absorbed fucoxanthin metabolites accumulated in the visceral white adipose tissue, and this preference for uptake into adipose tissue was supported by another mice study to a lesser degree (Yonekura et al. 2010). This is largely due to how fucoxanthin is absorbed, as it is a fat-soluble compound that is incorporated into mixed micelles in the small intestine before being absorbed similar to dietary lipids (Miyashita & Hosokawa 2017). For this reason, fucoxanthin administered in combination with medium-chain triglycerides (MCT) had a greater anti-obesity effect than fucoxanthin alone, likely due to facilitating absorption (Maeda, Hosokawa, Sashima, Funayama, et al. 2007). Combining fucoxanthin and edible oils has the additional benefit of helping to stabilise fucoxanthin, which is highly susceptible to oxidation and isomerisation in response to light exposure, heat and oxygen (Zhao et al. 2014).

The present study used a 16-week rat model of diet-induced metabolic syndrome (Panchal, Poudyal, Iyer, Nazer, Alam, Diwan, Kauter, Sernia, Campbell, Ward, et al. 2011) to determine whether fucoxanthin extracted from *P. tricornutum* can reduce obesity, cardiovascular damage or liver changes. There has been very limited testing

of fucoxanthin in models of diet-induced obesity, particularly in rats, and consequently the optimal dose of fucoxanthin is not known. It is important to determine the optimal dose in animal studies before progressing to clinical trials, as this helps to ensure the greatest chance of success in translating results. A dose of 1 mg/kg body weight/day in rats reduced intra-abdominal fat, serum lipids, blood glucose and blood pressure, but was unable to reduce retroperitoneal fat or total body weight (Grasa-López et al. 2016). Similar to this, a low dose of 0.65 mg/kg body weight and a higher dose of fucoxanthin of 6.5 mg/kg body weight were selected for this study to further explore the effect of reducing or substantially increasing the dose of fucoxanthin given to rats. These doses were selected as they equate to a dose of 10 mg/day and 100 mg/day for an obese human weighing 100 kg (Reagan-Shaw, Nihal & Ahmad 2008). In response to prior evidence that combining fucoxanthin with edible oils increased bioavailability and anti-obesity effects, the fucoxanthin used in this study was provided in combination with MCT oil. The hypothesis for this study was that the high dose of fucoxanthin would reduce abdominal fat, total body weight and cardiovascular changes in diet-induced metabolic syndrome. It was predicted that the major mechanism of these effects would be a lipolytic effect of fucoxanthin to increase fat oxidation and consequently reduce storage in adipose tissue depots. The low dose will be able to provide evidence on whether 1 mg/kg body weight/day is the lowest effective dose in rats.

4.2 Methods and Materials

The materials and methods have been described in section CHAPTER 2 - MATERIALS AND METHODS with the following modifications.

4.2.1 Fucoxanthin production and analysis

Biomass of *P. tricornutum* was cultivated by PacificBio at a land-based aquaculture facility near Ayr, Queensland, Australia (19°29’S, 147°28E). A fucoxanthin oleoresin was extracted from *P. tricornutum* by PacificBio using an extraction process that is protected as intellectual property. Analysis was conducted by PacificBio to determine the concentrations of fucoxanthin, lipids, proteins, carbohydrates, heavy metals, vitamins and other components in the oleoresin. The fucoxanthin oleoresin was stored

at -20 °C and combined with MCT oil to improve solubility and bioavailability when combining fucoxanthin with the high-carbohydrate, high-fat (HCHF) and corn starch (CS) diets. The MCT oil was 100% from coconut oil and consisted of caprylic acid 55-70%, capric acid 29-45 %, and less than 2% caproic acid and 3% lauric acid. The fucoxanthin-MCT mixture that was administered in the HCHF and CS diet contained 50% fucoxanthin oleoresin and 50% MCT oil.

4.2.2 Diet regimes

Rats (n = 72, weighing 338 ± 1 g) were randomly distributed into six experimental groups (n = 12 rats/group) as shown in Table 23.

Table 23: Treatment groups diet regime

Treatment Group	0 – 8 Week Diet	8 – 16 Week Diet
C	CS	CS
CFL	CS	CS + 0.65 mg/kg body weight/day fucoxanthin
CFH	CS	CS + 6.5 mg/kg body weight/day fucoxanthin
H	HCHF	HCHF
HFL	HCHF	HCHF + 0.65 mg/kg body weight/day fucoxanthin
HFH	HCHF	HCHF + 6.5 mg/kg body weight/day fucoxanthin

The fucoxanthin treatment diets were prepared by gently heating the fucoxanthin-MCT mixture to 40 °C using a baby bottle warmer (Tommee Tippee, Mount Waverley, Victoria, Australia) to bring the mixture to a liquid form. The results of the fucoxanthin-MCT analysis determined that the mixture contained 2% fucoxanthin, and this was used to guide how much fucoxanthin-MCT mixture to add into the treatment diets to obtain the desired doses. The average daily food consumption of CS and HCHF rats observed in the ulvan and astaxanthin studies was also used to guide this calculation. Fucoxanthin is an unstable compound, so the treatment food in each individual bowl was emptied completely and refilled every day. The prepared

treatment diets were stored in an airtight container in the dark and were kept for no longer than three days to limit exposure to oxygen and light.

4.2.3 Cholesterol analysis

The serum low-density lipoprotein (LDL)/very-low density lipoprotein (VLDL) and high-density lipoprotein (HDL) were determined using a cholesterol assay kit (ab6539, Abcam, Cambridge, United Kingdom) following manufacturer instructions.

4.2.4 Adipocyte histology

Retroperitoneal fat was weighed after euthanasia and approximately 1 cm^3 of fat tissue per rat (4 rats per group) was stored in 10% formalin at 4 °C for 72 hours. The fat tissues were then transferred to 70% ethanol and stored at 4 °C for at least 48 hours before tissue processing and embedding in paraffin wax.

Tissue sectioning and preparation for staining was performed as described in section 2.4.2 Histology with the following modifications. After dehydration as discussed previously, samples underwent the following sequence: haematoxylin (2 minutes), running water (2 minutes), eosin Y (dip 12 times), distilled water (dip until eosin stops streaking), 50% ethanol (dip 10 times), 70% ethanol (dip 10 times), 95% ethanol (30 seconds), 100% ethanol (1 minute), and 100% xylene (1 minutes, 3 times). After staining, slides were fixed with a coverslip using DPX Mountant for histology. Adipose tissue sections were imaged at 20x magnification using a EVOS FL Color Imaging System on the standard white light setting.

Adipose tissue images taken with the microscope camera were then analysed using Image J bundled with Java version 1.8.0_172 (National Institutes of Health, Bethesda, Maryland, United States) with computer analysis as described by Parlee et al. (2014). The data generated from this method included the size and number of adipocytes within the 20x magnification image of the retroperitoneal fat, and this data was analysed as described in section 2.6 Statistical Analysis.

4.2.5 Dual-energy x-ray absorptiometry

The Norland XR46 DXA instrument was not functioning at the time that the 16-week study was ending, and consequently body composition was not able to be tested for this study.

4.2.6 Gut microbiome

Analysis was conducted as described in section 2.5 Gut microbiota analysis with the following modification. The analysis was completed by Dr Marwan Majzoub at the University of New South Wales instead of Dr Priscila Goncalves.

4.3 Results

4.3.1 Fucoxanthin composition

Fucoxanthin oleoresin was analysed to determine the composition (Table 24).

Table 24: Fucoxanthin oleoresin analysis

Biochemical Composition		Oleoresin % dry weight
Fucoxanthin		4.3
Other pigments		1.2
Moisture		12
Ash		2.3
Proteins		2.7
Carbohydrates		10.1
Total lipids		67.5
Total saturated fatty acids	16.3	
Total mono-unsaturated fatty acids	14.2	
Total poly-unsaturated fatty acids	36.9	
Saturated fatty acids		g/100 g
C16:0 Palmitic acid		9.2
C14:0 Myristic acid		5.4
C24:0 Lignoceric		1.0
Mono-unsaturated fatty acids		g/100 g
C16:1(n-7) Palmitoleic acid		12.6
C18:1(n-9 or n-7) Oleic acid		0.9
Poly-unsaturated fatty acids		g/100 g
Total omega 3 fatty acids		32.5
C20:5(n-3) Eicosapentaenoic		29.7
C18:3(n-3) alpha-Linolenic		1.5
Total omega 6 fatty acids		4.4
C18:2(n-6) Linoleic		3.4
C20:4(n-6) Arachidonic		0.6
Heavy metals		mg/kg
Mercury (Hg)		< 0.01
Cadmium (Cd)		< 0.01
Arsenic (As)		4.23
Nickel (Ni)		1.27
Lead (Pb)		0.03
Vitamins		
Retinol (vitamin A)		< 5 µg/100 g
Ergocalciferol (vitamin D2)		< 0.5 µg/100 g
Pyridoxine (vitamin B6)		0.05 mg/100 g

4.3.2 Physiology and metabolic indicators

HCHF diet-fed rats consumed less food than CS diet-fed rats. However, due to the energy density of the HCHF diet, they consumed on average 75 more kilojoules of energy per day than CS rats (Table 25). The measurements at 8 weeks confirmed that HCHF diet-fed rats had a higher body weight and fat mass compared to CS diet-fed rats (Figure 35 and Table 25). The difference in body weight between the two diets continued to increase in the following 8 weeks of the study. Fucoxanthin at both low and high dose did not change body weight, nor did it affect food or water intake. A dose of 0.65 and 6.5 mg/kg body weight/day was the aim of this study, however due to the rats consuming more than expected, HUL and HUH rats received a dose of 0.79 and 7.48 mg/kg body weight/day respectively. The CS diet has a lower energy density, and consequently rats consume more food on this diet than the HCHF diet, and this led to the CUL and CUH rats receiving a dose of 1.75 and 17.12 mg/kg body weight/day respectively.

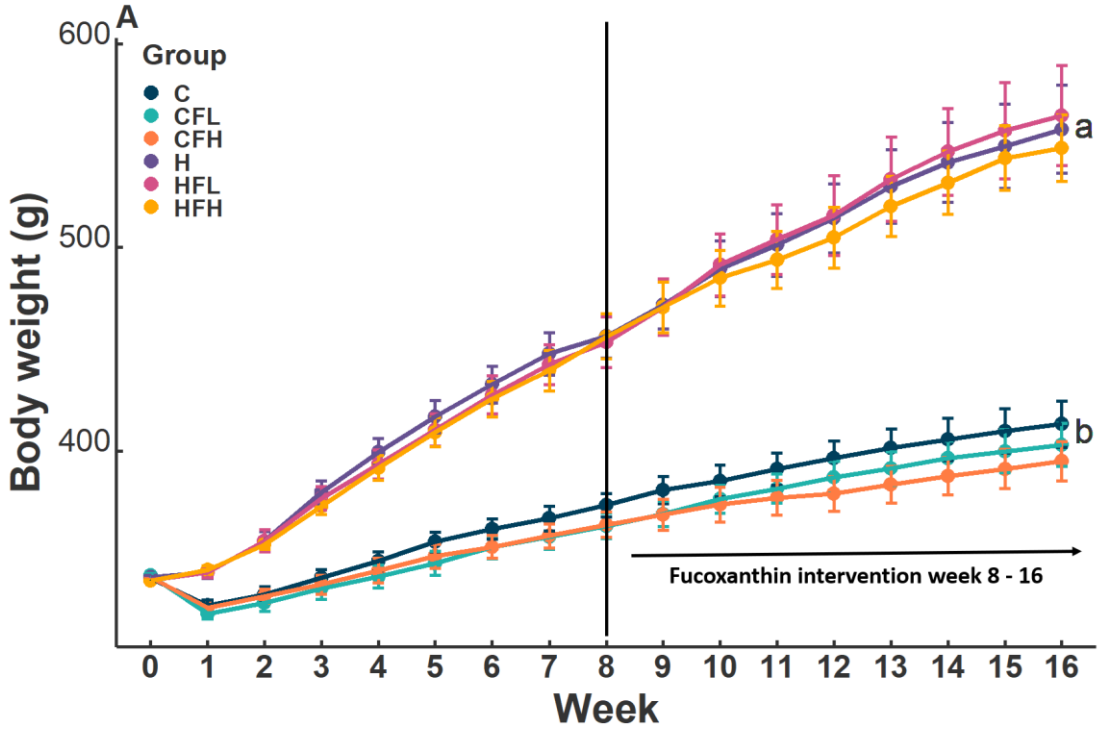


Figure 35: Body weight 0 – 16 weeks. Values are mean ± SEM, n = 12. Means without a common letter differ, $p < 0.05$. C, corn starch diet-fed rats; CFL, corn starch diet-fed rats supplemented with low dose fucoxanthin; CFH, corn starch diet-fed rats supplemented with high dose fucoxanthin; H, high-carbohydrate, high-fat diet-fed rats; HFL, high-carbohydrate, high-fat diet-fed rats supplemented with low dose fucoxanthin; HFH, high-carbohydrate, high-fat diet-fed rats supplemented with high dose fucoxanthin.

After 8 weeks, HCHF diet-fed rats had developed a reduced tolerance to an oral glucose load compared to CS rats (Table 25). The response to the glucose tolerance test at week 16 is displayed in Figure 36. There was no difference in response in glucose concentration at the different time points, and the area under the curve (AUC) was not different. From 8 – 16 weeks, the AUC of fucoxanthin-fed rats decreased whereas the HCHF diet-fed rats increased during this period (Figure 36B). This difference between H and HFH neared significance (p = 0.06).

HCHF diet-fed rats had higher total abdominal fat than CS rats (Table 25) and the size of adipocytes in the retroperitoneal fat also increased (Figure 37A). The frequency of adipocyte size as shown in Figure 37B highlights that 83% of CS rat adipocytes range from 1 – 6 10^3 x μm^2 whereas HCHF rats have more of a spread distribution. For CS diet-fed rats, 25% of all adipocytes in the retroperitoneal fat were less than 2 x 10^3 x μm^2 and 50% were less than 3 x 10^3 x μm^2 (Figure 37C). For HCHF diet-fed rats, 25% of all adipocytes in the retroperitoneal fat were less than 4 x 10^3 x μm^2 and 50% were less than 8 x 10^3 x μm^2 (Figure 37C).

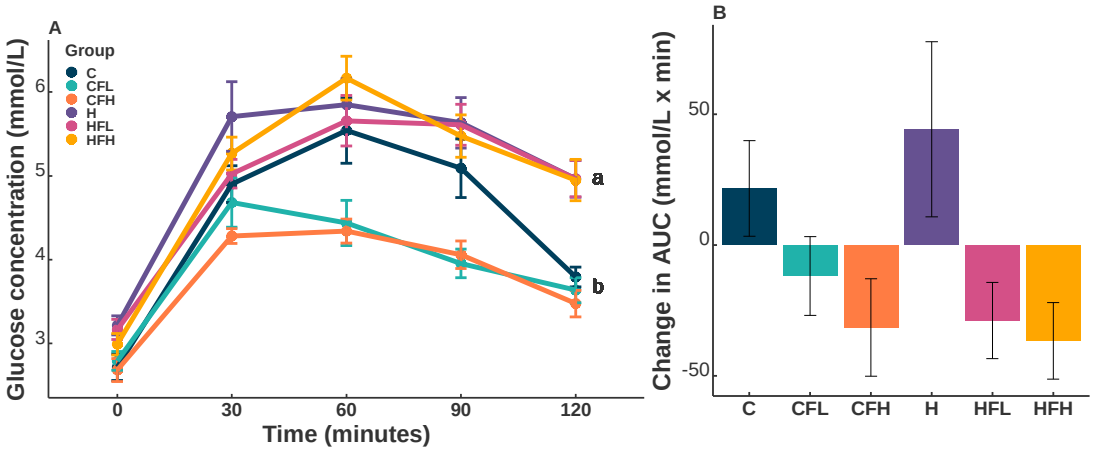


Figure 36: Results of the glucose tolerance test. ***A*** *Oral glucose tolerance test results at week 16.* ***B*** *Change in area under the curve from 8–16 week glucose tolerance test. Values are mean ± SEM, n = 12. Means without a common letter differ, p < 0.05. C, corn starch diet-fed rats; CFL, corn starch diet-fed rats supplemented with low dose fucoxanthin; CFH, corn starch diet-fed rats supplemented with high dose fucoxanthin; H, high-carbohydrate, high-fat diet-fed rats; HFL, high-carbohydrate, high-fat diet-fed rats supplemented with low dose fucoxanthin; HFH, high-carbohydrate, high-fat diet-fed rats supplemented with high dose fucoxanthin.*

Table 25: Physiological parameters of rats fed control, low dose and high dose fucoxanthin.

Variable	C	CFL	CFH	H	HFL	HFH	Diet	Treatment	Interaction
Body weight (g) week 8	373 ± 6^{b}	363 ± 6^{b}	364 ± 6^{b}	457 ± 11^{a}	453 ± 12^{a}	456 ± 11^{a}	< 0.001	0.757	0.871
Body weight (g) week 16	413 ± 11^{b}	403 ± 11^{b}	395 ± 10^{b}	558 ± 22^{a}	565 ± 25^{a}	549 ± 16^{a}	< 0.001	0.667	0.881
Body weight gain %	10.6 ± 1.8^{b}	10.9 ± 1.9^{b}	8.5 ± 1.2^{b}	21.9 ± 2.0^{a}	24.2 ± 2.6^{a}	20.2 ± 1.1^{a}	< 0.001	0.219	0.855
Water intake (g/day) week 0-8	35.0 ± 2.2^{a}	32.0 ± 2.5^{ab}	30.7 ± 1.8^{ab}	27.6 ± 1.1^{b}	27.2 ± 0.8^{b}	26.8 ± 0.9^{b}	< 0.001	0.275	0.550
Water intake (g/day) week 8-16	24.9 ± 1.9	26.2 ± 1.3	22.9 ± 1.5	24.8 ± 0.9	25.9 ± 0.8	25.1 ± 0.4	0.497	0.263	0.548
Food intake (g/day) week 0-8	42.2 ± 0.6^{a}	42.8 ± 0.6^{a}	42.1 ± 0.8^{a}	25.6 ± 0.9^{b}	24.7 ± 0.6^{b}	23.9 ± 0.8^{b}	< 0.001	0.432	0.479
Food intake (g/day) week 8-16	40.5 ± 0.5^{a}	41.1 ± 0.6^{a}	39.8 ± 0.9^{a}	24.5 ± 0.7^{b}	24.7 ± 0.9^{b}	22.9 ± 0.8^{b}	< 0.001	0.126	0.860
Energy intake (kJ/day) week 0-8	473 ± 7^{b}	481 ± 7^{b}	473 ± 9^{b}	565 ± 18^{a}	546 ± 11^{a}	530 ± 12^{a}	< 0.001	0.294	0.301
Energy intake (kJ/day) week 8-16	452 ± 7^{b}	461 ± 6^{b}	447 ± 10^{a}	533 ± 15^{a}	540 ± 15^{a}	506 ± 14^{b}	< 0.001	0.116	0.570
Feed efficiency week 0-8	0.08 ± 0.01^{b}	0.05 ± 0.01^{b}	0.06 ± 0.0^{b}	0.21 ± 0.02^{a}	0.21 ± 0.02^{a}	0.22 ± 0.02^{a}	< 0.001	0.680	0.505
Feed efficiency week 8-16	0.09 ± 0.01^{b}	0.09 ± 0.01^{b}	0.07 ± 0.0^{b}	0.19 ± 0.02^{a}	0.20 ± 0.02^{a}	0.18 ± 0.01^{a}	< 0.001	0.459	0.891
Fucoxanthin intake (mg/kg body weight/day)	0.00 ± 0.00^{d}	1.75 ± 0.04^{c}	17.12 ± 0.38^{a}	0.00 ± 0.00^{d}	0.79 ± 0.03^{c}	7.48 ± 0.17^{b}	< 0.001	< 0.001	< 0.001
Systolic blood pressure week 8 (mmHg)	125 ± 3^{b}	134 ± 5^{ab}	127 ± 3^{ab}	137 ± 4^{ab}	141 ± 4^{ab}	141 ± 4^{a}	0.002	0.304	0.701
Systolic blood pressure week 16 (mmHg)	131 ± 4^{ab}	128 ± 4^{ab}	129 ± 5^{ab}	146 ± 5^{a}	126 ± 2^{b}	130 ± 5^{ab}	0.207	0.039	0.149

Table 26: Physiological parameters of rats fed control, low dose and high dose fucoxanthin continued.

Variable	C	CFL	CFH	H	HFL	HFH	Diet	Treatment	Interaction
Tissue wet weight (mg/mm tibial length)									
Retroperitoneal fat	233 ± 23^{b}	194 ± 26^{b}	195 ± 16^{b}	616 ± 57^{a}	609 ± 72^{a}	569 ± 46^{a}	< 0.001	0.641	0.892
Omental fat	164 ± 16^{b}	144 ± 14^{b}	133 ± 13^{b}	342 ± 34^{a}	365 ± 41^{a}	344 ± 29^{a}	< 0.001	0.812	0.730
Epididymal fat	113 ± 14^{b}	87 ± 13^{b}	93 ± 9^{b}	249 ± 30^{a}	298 ± 38^{a}	250 ± 24^{a}	< 0.001	0.649	0.271
Total abdominal fat	507 ± 55^{b}	425 ± 50^{b}	420 ± 37^{b}	1133 ± 92^{a}	1272 ± 143^{a}	1162 ± 95^{a}	< 0.001	0.795	0.464
Kidneys	51 ± 1	55 ± 2	63 ± 11	59 ± 2	61 ± 1	58 ± 1	0.404	0.564	0.407
Liver	248 ± 11^{b}	241 ± 10^{b}	238 ± 5^{b}	378 ± 23^{a}	387 ± 13^{a}	361 ± 8^{a}	< 0.001	0.446	0.656
Left ventricle and septum	23.6 ± 0.7^{b}	23.2 ± 0.8^{b}	24.2 ± 0.9^{b}	25.2 ± 2.1^{ab}	25.3 ± 0.9^{a}	26.5 ± 0.9^{ab}	0.013	0.468	0.944
Right ventricle	4.7 ± 0.2^{b}	4.7 ± 0.3^{b}	4.6 ± 0.3^{b}	5.9 ± 0.5^{ab}	6.1 ± 0.5^{a}	5.8 ± 0.3^{ab}	< 0.001	0.756	0.956
Serum parameters									
ALT (U/L)	29.6 ± 4.6^{ab}	22.3 ± 1.0^{b}	27.6 ± 1.5^{ab}	30.2 ± 2.3^{ab}	27.7 ± 1.8^{ab}	34.0 ± 2.2^{a}	0.053	0.056	0.475
AST (U/L)	143.1 ± 8.8	151.9 ± 9.8	145.1 ± 12.0	131.4 ± 11.8	145.4 ± 11.1	172.0 ± 8.7	0.675	0.138	0.138
LDL and VLDL (µg/µl)	0.45 ± 0.04^{b}	0.30 ± 0.04^{b}	0.90 ± 0.08^{a}	0.53 ± 0.07^{b}	0.41 ± 0.06^{b}	1.15 ± 0.13^{a}	0.018	< 0.001	0.528
HDL (µg/µl)	0.34 ± 0.04^{b}	0.40 ± 0.05^{b}	0.36 ± 0.02^{b}	0.42 ± 0.04^{b}	0.47 ± 0.07^{b}	0.82 ± 0.05^{a}	< 0.001	< 0.001	< 0.001
Oral glucose tolerance test									
AUC week 8 (mmol/L x min)	528 ± 17^{b}	508 ± 21^{b}	504 ± 18^{b}	604 ± 18^{a}	627 ± 14^{a}	663 ± 18^{a}	< 0.001	0.532	0.069
AUC week 16 (mmol/L x min)	564 ± 26^{ab}	489 ± 19^{b}	473 ± 13^{b}	648 ± 35^{a}	611 ± 23^{a}	627 ± 21^{a}	< 0.001	0.030	0.343

Differing lower-case superscript letters indicate significant difference at $p<0.05$ determined by Tukey's HSD. ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the curve; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; HDL, high-density lipoprotein;

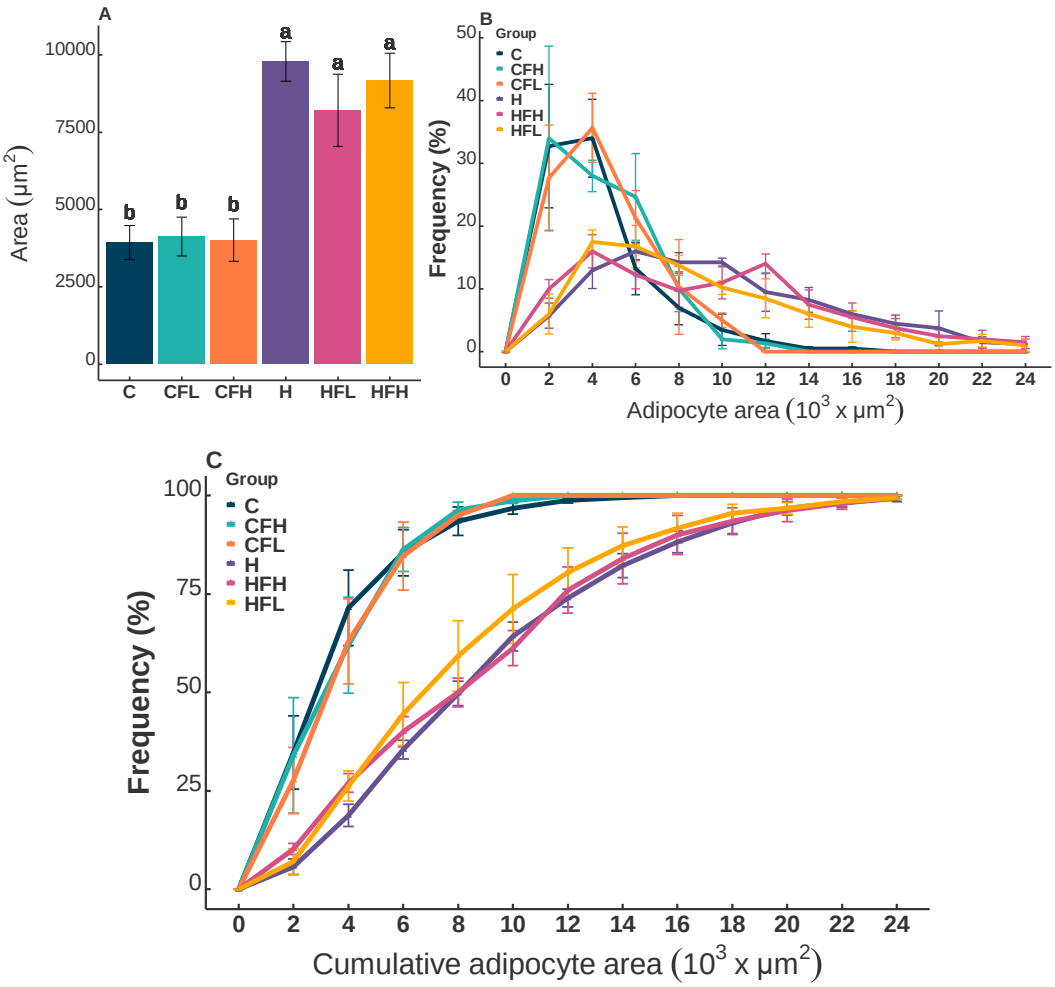


*Figure 37: Retroperitoneal adipocyte histology analysis at week 16. Values are mean ± SEM, n = 4. Means without a common letter differ, $p < 0.05$. **A** Average adipocyte area. **B** Frequency of adipocyte size. **C** Cumulative frequency of adipocyte size. C, corn starch diet-fed rats; CFL, corn starch diet-fed rats supplemented with low dose fucoxanthin; CFH, corn starch diet-fed rats supplemented with high dose fucoxanthin; H, high-carbohydrate, high-fat diet-fed rats; HFL, high-carbohydrate, high-fat diet-fed rats supplemented with low dose fucoxanthin; HFH, high-carbohydrate, high-fat diet-fed rats supplemented with high dose fucoxanthin*

4.3.3 Serum lipids

After 16 weeks, the HCHF diet increased serum triglycerides but did not affect HDL or LDL/very low-density lipoprotein (VLDL) concentrations (Figure 38). High dose fucoxanthin reduced triglyceride concentrations to be equal to CS diet-fed rats and increased both HDL and LDL/VLDL concentration in comparison to all groups. No changes in the low dose fucoxanthin groups were observed in comparison to controls.

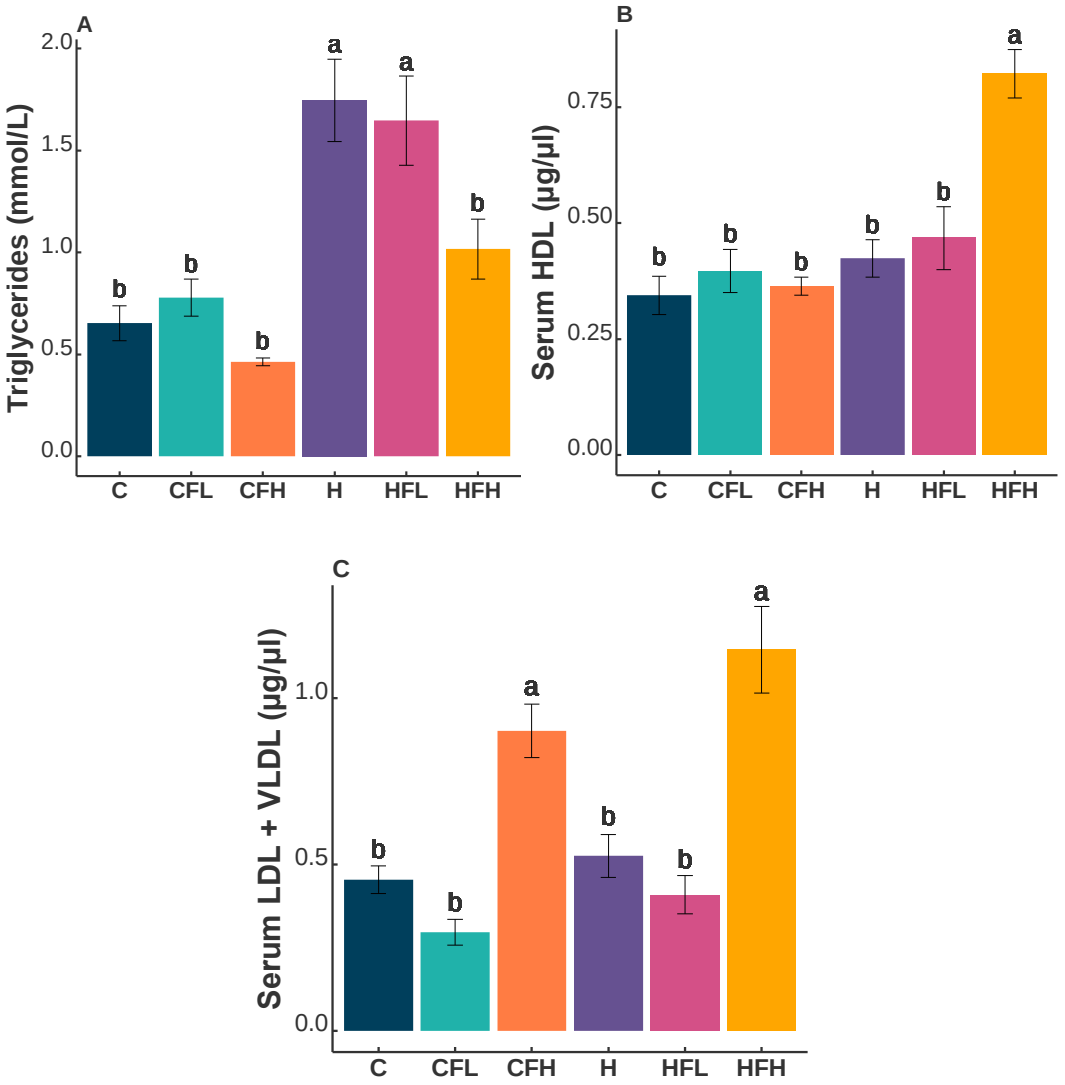


*Figure 38:Serum lipids analysed at week 16. **A** Triglycerides. **B** High-density lipoprotein. **C** Low-density lipoprotein. Values are mean ± SEM, n = 12. Means without a common letter differ, p < 0.05. C, corn starch diet-fed rats; CFL, corn starch diet-fed rats supplemented with low dose fucoxanthin; CFH, corn starch diet-fed rats supplemented with high dose fucoxanthin; H, high-carbohydrate, high-fat diet-fed rats; HFL, high-carbohydrate, high-fat diet-fed rats supplemented with low dose fucoxanthin; HFH, high-carbohydrate, high-fat diet-fed rats supplemented with high dose fucoxanthin.*

4.3.4 Liver structure and systolic blood pressure

The total wet liver weight was higher in HCHF diet-fed rats than CS diet-fed rats, but there was no change in fucoxanthin treatment groups in comparison to controls (Table 25). Livers from H rats showed increased fat deposition causing hepatocyte ballooning compared to C rats (Figure 39). In the four rats per group that were analysed, the

average score of liver fat deposition out of a maximum of 5 was 0.5 (C), 0 (CFL), 0 (CFH), 4.5 (H), 3.6 (HFL) and 3.5 (HFH). Hence, lower for both HFL and HFH compared to H. Liver inflammatory cell infiltration varied between groups. There was no difference in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities between H and C rats, although HFH ALT was higher than CFL ALT (Table 25).

Systolic blood pressure was measured at both 8 and 16 weeks, and during this period both C and H increased, and the fucoxanthin-fed groups decreased (Figure 40). At week 16, HFL rats had lower systolic blood pressure than H rats and HFH was nearing significant difference ($p = 0.18$) (Table 25).

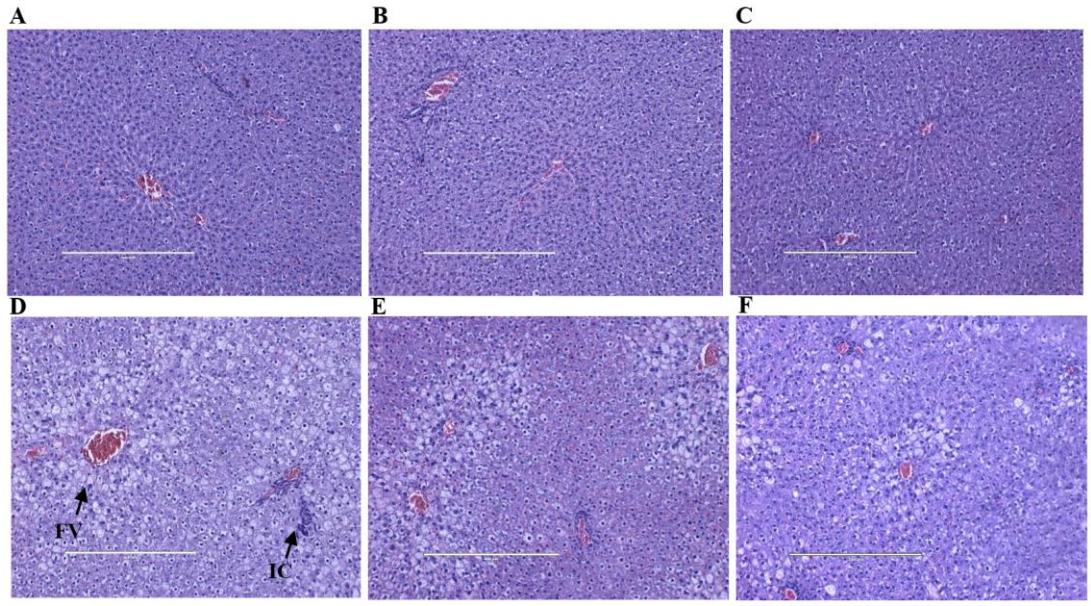


Figure 39: Haematoxylin and eosin staining of the liver to show fat deposition and inflammatory cell infiltration. Fat vacuoles are marked as 'FV' and inflammatory cells are marked as 'IC'. Scale = 400 µm. ***A*** *C liver.* ***B*** *CFL liver.* ***C*** *CFH liver.* ***D*** *H liver.* ***E*** *HFL liver.* ***F*** *HFH liver. An enlarged image of H, HFL and HFH liver can be found in Supplementary Figure 2.*

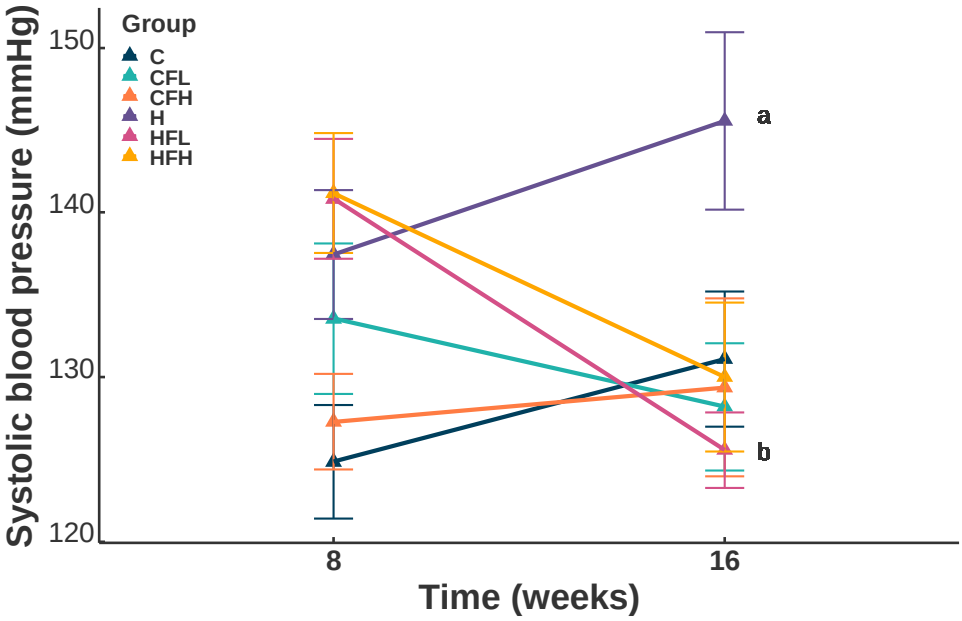


Figure 40: Systolic blood pressure comparison from 8-16 week. Values are mean ± SEM, n = 6-12. Means without a common letter differ, p < 0.05 ('a' is referring to H and 'b' is referring to HFL). C, corn starch diet-fed rats; CFL, corn starch diet-fed rats supplemented with low dose fucoxanthin; CFH, corn starch diet-fed rats supplemented with high dose fucoxanthin; H, high-carbohydrate, high-fat diet-fed rats; HFL, high-carbohydrate, high-fat diet-fed rats supplemented with low dose fucoxanthin; HFH, high-carbohydrate, high-fat diet-fed rats supplemented with high dose fucoxanthin.

4.3.5 Gut microbiome

The gut microbiota was different between C samples and H samples without fucoxanthin (p = 0.002), indicating an effect of diet on the bacterial community structure (Figure 41A). There was also a statistically significant effect of the addition of fucoxanthin to the C diet (p = 0.0387), but not for the H diet (p = 0.6578). There were no changes in Shannon's diversity or richness for both diet and fucoxanthin intake (Figure 41B and 41C). The HCHF diet-fed rats had an increase in the Firmicutes/Bacteroidetes (F/B) ratio compared to the CS diet-fed rat samples (p = 0.0011) (Figure 41D). There was no impact of fucoxanthin supplementation on the F/B ratio.

The most abundant bacterial classes found in the faecal samples for different treatment groups belonged to the classes Bacteroidia, Bacilli, Clostridia, Erysipelotrichia and Verrucomicrobiae (Figure 42A). An increase in the relative abundance of bacteria

from the class Erysipelotrichia was observed in the C samples compared to the H samples, while an increase was observed in HFH samples compared to the CFH samples. An increase in the relative abundance of bacteria from the class Bacteroidia and Verrucomicrobiae was observed in the C diet samples compared to the H diet samples (Figure 42A). In contrast, there was an increase in the relative abundance of bacteria from the class Bacilli and Clostridia in the H diet samples compared to the C diet samples.

An increase in the relative abundance of bacteria from the families *Bacteroidaceae*, *Prevotellaceae* and *Akkermansiacaeae* was observed in the C diet samples compared to the H diet samples (Figure 42B). In contrast, an increase in the relative abundance of bacteria from the families *Lactobacillaeceae*, *Peptostreptococcaceae* and *Ruminococcaceae* was observed in the H diet samples compared to the C diet samples. Relative abundance of bacteria in the family *Muribaculaceae* in the C diet samples was increased compared to the H diet samples. Similarly, there was a significant increase in the relative abundance of bacteria from the family *Lachnospiraceae* in the H diet samples compared to the C diet samples (Figure 42B). There was no impact of fucoxanthin supplementation on the taxonomic structure of the bacterial communities.

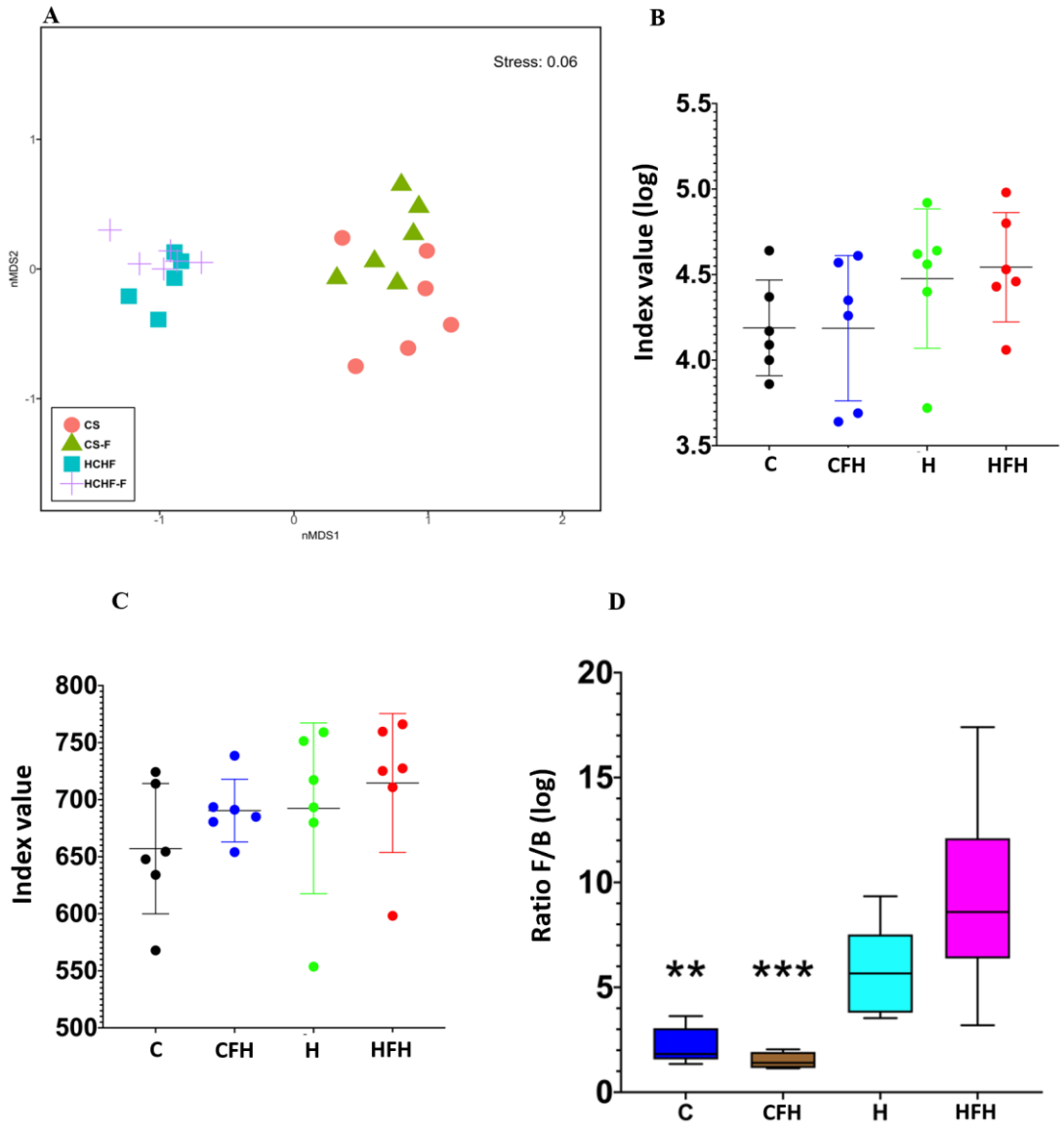


*Figure 41: Effect of dietary supplementation of fucoxanthin on gut microbiota. **A** Bacterial community structure of rat faeces under different diets. Non-metric multidimensional scaling (NMDS) plots incorporating all OTUs generated (n = 1200). In these plots, each point corresponds to one sample and incorporates the abundance of all OTUs present in that sample. **B** Shannon diversity of faecal samples. **C** Richness of faecal samples. **D** Effect of diet and fucoxanthin on the ratio of Firmicutes and Bacteroidetes abundance. The ** denotes a significant difference of C compared to H ($p < 0.01$) and ** denotes a significant difference of CFH compared to H ($p < 0.001$).*

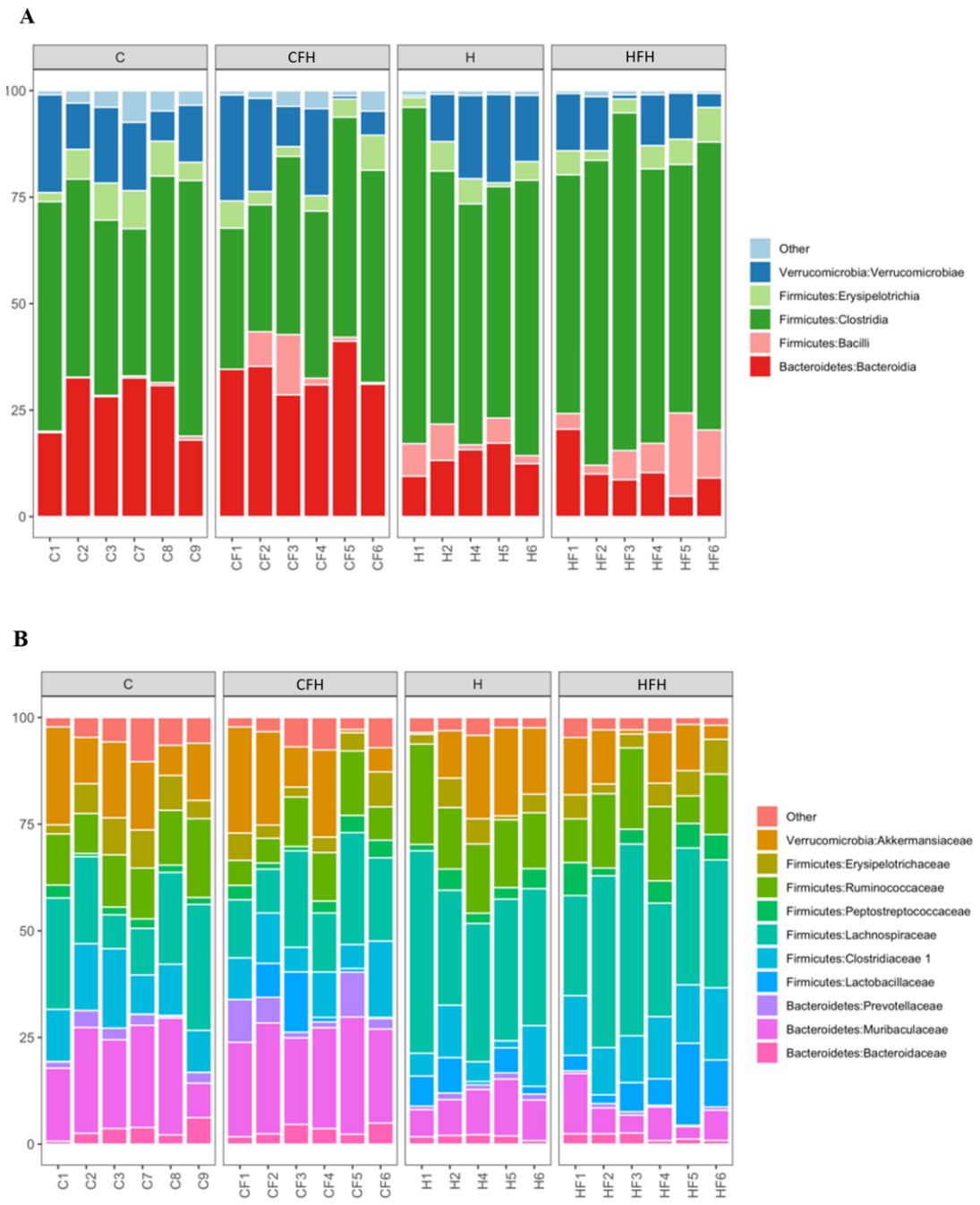


*Figure 42: Taxonomic structure of the bacterial communities **A** Taxonomic profiles of bacterial communities shown at the class level of all faecal samples. **B** Taxonomic profiles of bacterial communities shown at the family level of all faecal samples.*

4.4 Discussion

In this study, the HCHF diet led to a phenotype associated with diet-induced metabolic syndrome including obesity, metabolic abnormalities, hypertension and ectopic fat deposition in the liver. This change was associated with an increase in the F/B ratio; however, overall species diversity was unchanged. Introducing fucoxanthin into the HCHF-diet normalised systolic blood pressure and reduced fat deposition in the liver. Further, the high dose fucoxanthin markedly reduced serum triglycerides and increased serum HDL and LDL/VLDL cholesterol. In contrast to the major findings in previous studies (Grasa-López et al. 2016; Hosokawa et al. 2010; Woo et al. 2009), fucoxanthin did not induce changes to abdominal fat deposition, adipocyte size or total body weight.

Fucoxanthin is a potent antioxidant that has previously demonstrated multiple bioactivities including anti-obesity (Woo et al. 2009), anti-cancer (Okuzumi et al. 1993), anti-diabetic (Maeda et al. 2015) and neuroprotection (Alghazwi et al. 2019). In previous rodent studies, fucoxanthin decreased body weight, fat deposition, cholesterol, triglycerides and blood pressure, and increased HDL-cholesterol and glucose tolerance (Grasa-López et al. 2016; Hosokawa et al. 2010; Maeda et al. 2015; Woo et al. 2009). These were prevention studies, whereby fucoxanthin was administered daily starting from the beginning of the study. This is a clear difference to the current study, which used a therapeutic intervention model, and this will be considered further later in this discussion. This reduction of obesity was translated to humans, although there has only been one small clinical trial thus far (Hitoe & Shimoda 2017).

Early investigation into mechanisms of action suggests that fucoxanthin is inducing this weight loss and other reductions in blood glucose and blood pressure through decreasing adipocytokines (inflammatory) and lipogenic enzymes, and upregulating uncoupling protein 1 (UCP1) expression to increase thermogenesis (Grasa-López et al. 2016; Hosokawa et al. 2010; Woo et al. 2009). Fucoxanthin has also shown promising anti-cancer effects in previous studies, including reducing the incidence of duodenal (Okuzumi et al. 1993) and sarcoma tumours (Wang, J et al. 2012) and delaying tumour growth and volume in another study (Ishikawa et al. 2008).

Fucoxanthin is believed to produce these anti-cancer effects through modulating protein levels and cellular signal transduction pathways (Satomi 2017).

This ability to modulate protein levels and gene expression appears to be the mechanism behind fucoxanthin's effect on serum lipids (Beppu et al. 2012). The average combined LDL and VLDL serum concentration for 20 week old Sprague Dawley albino male rats was 24 mg/dL (Ihedioha, Noel-Uneke & Ihedioha 2013), which is similar to the concentration found in this study in CS-diet fed Wistar rats (37 mg/dL). In comparison, the high dose of fucoxanthin increased the serum LDL/VLDL concentration in CFH rats to 90 mg/dL and in HFH rats to 115 mg/dL. It is well-accepted that high LDL-cholesterol increases the risk of atherosclerosis through causing endothelial dysfunction, formation of foam cells that are pro-inflammatory, and eventually increases the likelihood of plaque rupture and possible heart attack or stroke (Badimon & Vilahur 2012). Conversely, HDL-cholesterol is protective and reduces cardiovascular risk through removing cholesterol from the blood vessels and returning it to the liver to be excreted (Badimon & Vilahur 2012). The serum HDL-cholesterol concentration was increased in this current study from 42 mg/dL in the H rats to 82 mg/dL in the HFH rats. Low dose fucoxanthin did not change HDL-cholesterol or LDL/VLDL-cholesterol.

This effect of fucoxanthin elevating both LDL and HDL cholesterol concentrations has been observed in previous mice fucoxanthin studies at a dose of 500 mg/kg body weight and 0.2% diet (Beppu et al. 2012; Beppu et al. 2009). In these studies, they determined that this elevation was due to an upregulation of both sterol regulatory element-binding protein 1 and sterol regulatory element-binding protein 2, which are transcriptional factors involved in cholesterol synthesis (Beppu et al. 2012). Additionally, uptake and removal of LDL-cholesterol by the liver was reduced (Beppu et al. 2012). This would culminate in an increased serum LDL-cholesterol due to increased synthesis and reduced removal. This study did not investigate whether fucoxanthin affected triglyceride concentrations, as was observed in this current study. Fucoxanthin reduced triglycerides in a separate study conducted by Grasa-López et al. (2016). The mechanism behind this change was not explored, however it was theorised that this was also due to the modulation of genes involved in lipid metabolism (Grasa-López et al. 2016).

It should however be noted that the increase to LDL-cholesterol does not appear to be the case when fucoxanthin is provided at a lower dose. A dose of 1 mg/kg body weight in male Wistar rats reduced LDL/VLDL-cholesterol from 55 mg/dL in the control to 16 mg/dL in the fucoxanthin treatment group (Grasa-López et al. 2016). This is in line with the results of this current study, where the low dose used was 1.8 mg/kg body weight and 0.8 mg/kg body weight in the CFL and HFL rats respectively. In both of these low dose treatment groups, LDL/VLDL-concentration was trending towards a decrease compared to the control diets, and perhaps a higher dose or being provided as a prevention rather than as an intervention would have led to a significant decrease.

Despite the insignificant change in serum lipids in HFL rats, there was a 20 mm Hg reduction in systolic blood pressure from the 8^{th} week to the 16^{th} week with fucoxanthin treatment. There systolic blood pressure was trending towards being decreased in HFH rats during this same period, however this was not a significant change. The normalisation of systolic blood pressure by HFL rats is supported by the previous study by Grasa-López et al. (2016). There has been no prior research into the mechanisms of how fucocanthin reduces blood pressure; however there has been substantial evidence to show that eicosapentaenoic acid (EPA), included in the fucoxanthin oleoresin, can reduce blood pressure (Guo et al. 2019).

The fucoxanthin oleoresin used in the current study contained 29.7% EPA, although the final concentration when combined with MCT oil was approximately 15%. This equated to an approximate dose of 6.5 mg/kg body weight in HFL rats and 62 mg/kg body weight in HFH rats. The same model developed by Panchal et al. (2011) was used to test the effect of EPA at a dose of 700 mg/day, and this led to a reduction in systolic blood pressure (Poudyal et al. 2013). This far exceeded the dose of this current study, and therefore it is unlikely that this small amount of EPA produced the observed reduction to systolic blood pressure. Similarly, it is improbable that the coconut oil (MCT) used in this study would produce an effect on health, as the dose given was 100 mg/day in high dose rats and consequently far lower than the 5% diet dose used previously to reduce blood pressure in this same model (Panchal, Carnahan & Brown 2017).

It was theorised in the previous study by Grasa-López et al. (2016) that the reductions to the anti-inflammatory cytokine adiponectin and decreases in concentrations of pro-

inflammatory cytokines, C reactive protein and plasminogen activator inhibitor-1, were responsible for the blood pressure reduction. A future study utilising a model of Spontaneously Hypertensive Rats would be beneficial in determining whether fucoxanthin directly effects blood pressure or whether it is an indirect effect of another health change.

The algal carotenoid, astaxanthin, reduced systolic blood pressure in both humans (Mashhadi et al. 2018) and Spontaneously Hypertensive Rats (Monroy-Ruiz et al. 2011). There is evidence to suggest that the potent antioxidant effects of astaxanthin are responsible for this attenuation of hypertension (Monroy-Ruiz et al. 2011). Further investigation highlighted that astaxanthin reduced left ventricular weight and ameliorated vascular remodelling (Monroy-Ruiz et al. 2011), which has been observed after treatment with vitamins C and E, two other antioxidants (Chen et al. 2001). In a previous study in rats fed a high-fat diet, fucoxanthin reduced markers of oxidative stress and increased the activities of antioxidant enzymes (Ha, Na & Kim 2013). Therefore, it is possible that fucoxanthin induced this reduction to blood pressure in the current study through reducing oxidative stress. Further testing to analyse serum markers of oxidative stress and serum antioxidant enzymes would help to confirm whether this was either contributing or responsible for this normalisation of blood pressure.

The gut microbiome in this study was unchanged by fucoxanthin intake. There has been limited investigation into the effect of fucoxanthin on the gut microbiome, although one past study has observed that a dose of 50 mg/100 g diet and 100 mg/100 g diet in mice increased species diversity, prevalence of Bacteroidetes and decreased prevalence of Firmicutes (Sun et al. 2020a). Additionally, the serum lipopolysaccharide (bacterial endotoxin) was reduced in these mice, and the short-chain fatty acid butyrate was increased by the fucoxanthin dose of 100 mg/100g diet (Sun et al. 2020a). The high dose in this current study was far lower than the 100 mg/100 g dose in mice, as there was only 16.3 mg fucoxanthin/100 g of the rat HCHF diet, and it may be that a higher dose of fucoxanthin is required to alter the gut microbiome. The study by Sun et al. (2020) also observed a reduction to total body weight and adipocyte size, and so it may be that the gut microbiome change was instead due to the attenuation of obesity rather than a direct effect of fucoxanthin. It

has been observed that weight loss alone can increase species diversity and alter the abundance of specific bacterial genus' in clinical trials (Dhakal, McCormack & Dey 2020; Frost et al. 2019). More studies are required to determine whether there is a relationship between fucoxanthin and the gut microbiome.

In this study, deposition of fat in the liver was reduced by both the low and high fucoxanthin doses. Further investigation is necessary to quantify this change, and to determine whether it is triglycerides or cholesterol, or a combination of both, that have been reduced in the liver. It has been observed previously that fucoxanthin reduced cholesterol content in the liver, despite increasing overall liver weight (Beppu et al. 2012). This was the same study that observed that serum LDL-cholesterol and HDL-cholesterol were increased. It may be that reducing LDL-cholesterol receptors in the liver has led to a reduction in hepatic cholesterol content. However, it is difficult to determine why this change has occurred with the limited number of previous studies investigating the effect of fucoxanthin on hepatic fat deposition.

Alternatively, it may be the case that fat deposition in the liver was reduced due to an inhibitory effect of fucoxanthin on lipogenic enzyme activity and an upregulation of lipolysis as was previously observed by Woo et al. (2009). Whilst this effect of fucoxanthin is possible, it is unlikely in this current study as there was no change to body weight or abdominal fat as would be expected if this mechanism was occuring. The effect of fucoxanthin on body weight is controversial, as there are studies showing a beneficial effect (Beppu et al. 2012; Hosokawa et al. 2010; Woo et al. 2009) and studies showing no effect (Grasa-López et al. 2016; Hu et al. 2012; Maeda et al. 2015). Of these studies in mice, those that have shown a beneficial effect had a longer study duration of 4 – 6 weeks (Hosokawa et al. 2010; Woo et al. 2009) rather than 27 days (Maeda et al. 2015). Both studies in rats produced no change to total body weight but showed a decrease in abdominal fat deposition (Grasa-López et al. 2016; Hu et al. 2012). These studies used a lower dose than used previously in the mice studies, using 0.167 – 1 mg/kg body weight/day compared to 0.2% diet (Hosokawa et al. 2010; Woo et al. 2009). Whilst this current study was of a similar duration and dose used in these previous studies, it differs considerably in the design of the study.

The present study used a therapeutic intervention style model, whereby obesity is first induced over an 8-week period of HCHF diet-feeding, and then fucoxanthin is

introduced into that diet in the final 8 weeks in an attempt to reverse or mitigate the further development of obesity and other symptoms of metabolic syndrome. This model can be argued as being a more realistic test of the anti-obesity effects of fucoxanthin, as it replicates the typical progressive increase in body weight seen in humans due to an energy imbalance and the consequent attempt to reverse the previous weight gain. It has been observed that humans are more likely to seek out obesity treatments when they are already obese, as a positive relationship between body mass index and total obesity treatments sought was observed in a previous study (Ciao, Latner & Durso 2012). Further, if these anti-obesity effects are to be translated to humans, this will necessitate testing fucoxanthin in people who are already overweight or obese. Consequently, a therapeutic intervention style model such as the one used in this current study is an important challenge before progressing to clinical trials.

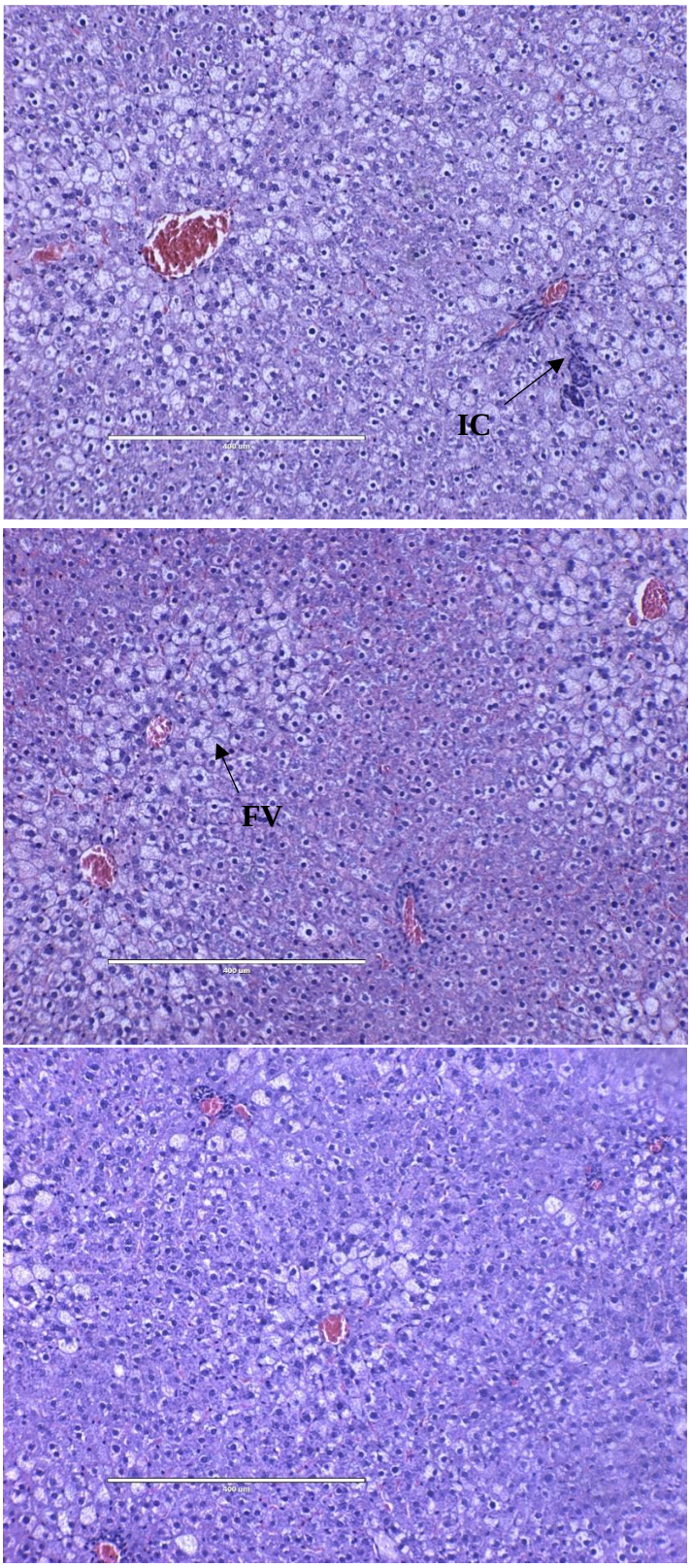
The lack of an anti-obesity effect shown in animal therapeutic intervention models may be a reason as to why there has thus far been only one clinical trial testing the effectiveness of fucoxanthin in obese patients (Hitoe & Shimoda 2017). This was a small (n=11 per group), placebo-controlled study that observed a beneficial effect of 1 mg/day fucoxanthin on body weight and abdominal fat. However, despite this success, in the following four years there has been no follow-up studies completed and only one clinical trial that is currently recruiting for a study of fucoxanthin's effect on metabolic syndrome in 28 participants (ClinicalTrials.gov 2020). This is in direct contrast to the multitude of clinical trials testing astaxanthin (Choi et al. 2011; Mashhadi et al. 2018; Ng et al. 2020; Xia et al. 2020); another promising antioxidant algal carotenoid. The doses used in this current study were selected as they equate to a dose of 10 mg/day and 100 mg/day for an obese human weighing 100 kg (Reagan-Shaw, Nihal & Ahmad 2008). This is in line with the future clinical trial (ClinicalTrials.gov 2020) that is proposing a fucoxanthin dose of 12 mg/day. It will be interesting to observe whether the results of the current study, namely the reduction to blood pressure by the low dose fucoxanthin (HFL), is translated to this study in humans.

In this current study, MCT-oil was used to solubilise the fucoxanthin oleoresin and to increase stability and bioavailability as shown previously (Maeda, Hosokawa, Sashima, Funayama, et al. 2007). This could be taken further by instead encapsulating

fucoxanthin using nanogels or nanoparticles. The potential of this has been shown previously, as chitosan nanogels increased the half-life of fucoxanthin 9-fold and the percent micellisation (simulated intestinal digestion) by 47% compared to control (Ravi & Baskaran 2015). Similarly, digestive stability and bioaccessibility were increased using chitosan-coated fucoxanthin nanoparticles in a simulated digestive model (Koo et al. 2016). Fucoxanthin is highly susceptible to oxygen and low pH, and consequently is likely to degrade during normal digestion (D'Orazio et al. 2012). If this could be circumvented using nanoparticles to prevent loss of fucoxanthin, this would greatly increase oral bioavailability and the potential for therapeutic use. These nanoparticles have yet to be tested *in vivo*, which would be the next step in testing the effectiveness of this delivery method. Nanoparticles are highly promising, as shown by a previous study that showed a 9-fold increase in oral bioavailability of encapsulated curcumin nanoparticles compared to curcumin in solution in the same rat model of diet-induced metabolic syndrome (Du Preez et al. 2019).

In conclusion, the results of this study provide evidence that fucoxanthin increases serum cholesterol and decreases blood pressure, serum triglycerides and hepatic fat deposition. No changes to body weight, abdominal fat deposition, the gut microbiome or glucose tolerance were observed. This was the first study that tested the effects of fucoxanthin in an intervention-style model, and this may have been why previously observed health benefits including reductions to body weight and abdominal fat deposition, modulation of the gut microbiome or improved glucose tolerance did not feature in this study.

4.5 Supplementary



Supplementary Figure 2: Haematoxylin and eosin staining of the liver to show fat deposition and inflammatory cell infiltration. Fat vacuoles are marked as 'FV' and inflammatory cells are marked as 'IC'. Scale = 400 µm. ***A*** *H liver.* ***B*** *HFL liver.* ***C*** *HFH liver.*

CHAPTER 5 – LOW DOSE ASTAXANTHIN IS NOT EFFECTIVE IN ATTENUATING SYMPTOMS OF METABOLIC SYNDROME IN RATS

5.1 Introduction

Astaxanthin is a carotenoid present in low concentrations in growing green algae that increases during hibernation in harsh environmental conditions to turn the microorganism red (Ambati et al. 2014). In algae, astaxanthin is found predominantly in the cytoplasm (approximately 99%) and this is believed to protect the nucleus and act as a light filter to reduce the risk of photodamage to the algae (Mascia et al. 2017). The richest source of astaxanthin is the microalgae *Haematococcus pluvialis* (Ambati et al. 2014), but only after *H. pluvialis* adopts a cyst cell structure in response to environmental stressors such as high light intensity (Figure 43) (Ho et al. 2018).

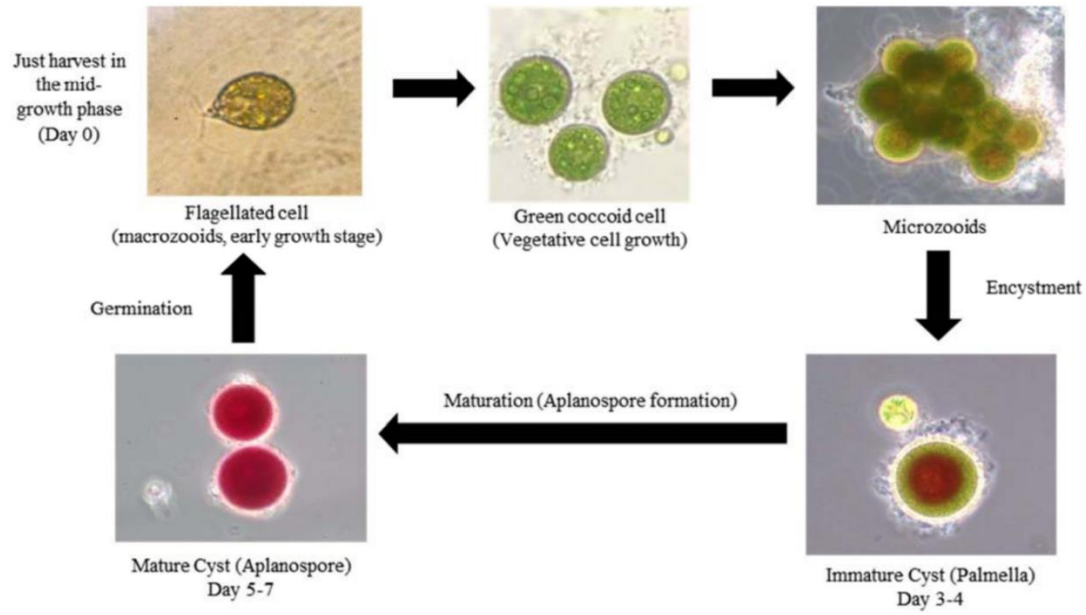


Figure 43: Photomicrograph of the life cycle of Haematococcus pluvialis (Ho et al. 2018).

Encystment of *H. pluvialis* involves a major shift in the cellular components, mainly the reduction in the photosynthetic chloroplasts and a marked increase in oil droplets containing astaxanthin (Figure 44) (Wayama et al. 2013).

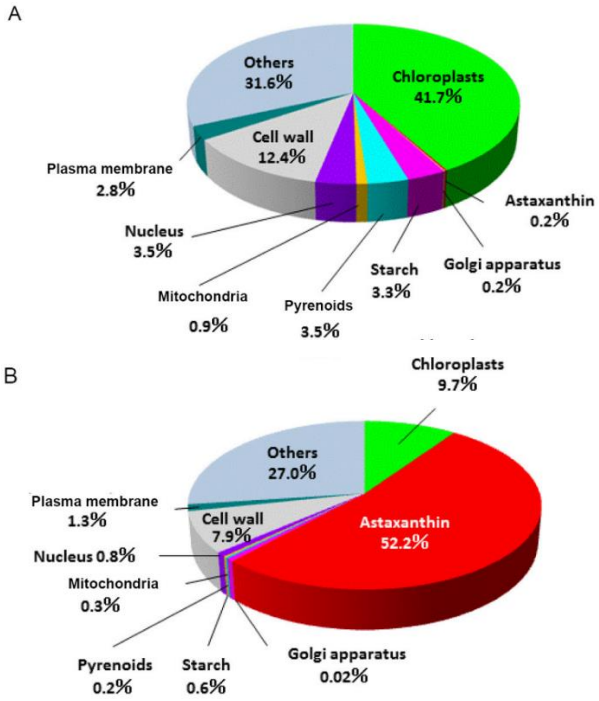


Figure 44: Relative volumes of Haematococcus pluvialis subcellular components. ***A****. Green coccoid cell.* ***B****. Red cyst cell (Wayama et al. 2013)*

Essentially, the microalgae goes into hibernation to survive the harsh environmental conditions, and purposely inducing this can yield approximately 415 mg fatty acids/g cell and 16.6 mg astaxanthin/g cell (Park, J-Y et al. 2020). Both of these are valuable commodities; fatty acids are sources for biodiesel production (Liu, J et al. 2016) and natural astaxanthin is used in dietary supplements, for personal care (skin-care), and in food and beverage and animal feed markets with a total market value of $600 million USD in 2018 (Ahuja & Rawat 2018). Only naturally produced astaxanthin can be used for direct human consumption, due to safety concerns of synthetic astaxanthin, and this is expected to bring an even greater dollar value due to growing public interest in natural products (Panis & Carreon 2016).

Carotenoids, including astaxanthin, are well-known for their antioxidant properties due to the conjugated double bonds within the hydrocarbon backbone allowing for potent free radical scavenging and singlet oxygen quenching activities (Figure 45)

(Dutta, Chaudhuri & Chakraborty 2005). Astaxanthin has more conjugated double bonds than other major carotenoids, and consequently astaxanthin is reported to be up to 10 times more potent as an antioxidant than β-carotene and lutein (Miki 1991; Naguib 2000). All-*trans* astaxanthin is the most commonly occurring isomer synthesised in *H. pluvialis (Brendler & Williamson 2019)*, and both the 9-*cis* and 13-*cis* isomers are found in natural astaxanthin (Brotosudarmo, Limantara & Setiyono 2020). There is evidence from *in vitro* studies that *cis* astaxanthin, particularly the 9-*cis* isomer, is more effective in reducing oxidative stress (Yang et al. 2017) and inflammation (Yang et al. 2019) than the all-*trans* form.

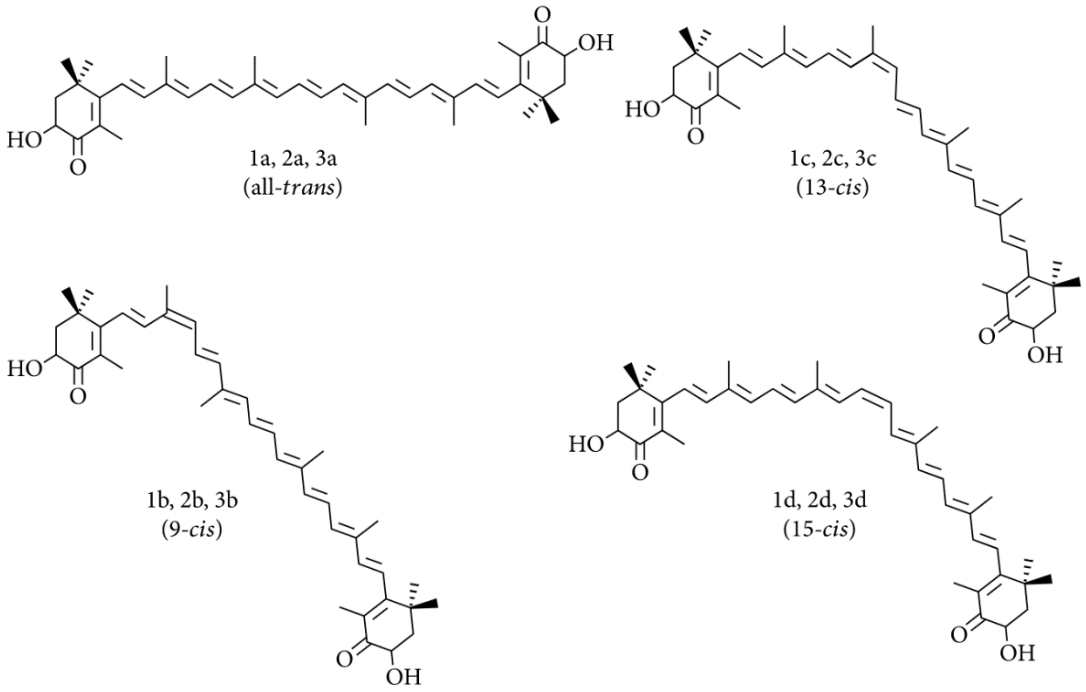


Figure 45: Structure of astaxanthin (Brotosudarmo, Limantara & Setiyono 2020).

Antioxidants protect against protein and lipid oxidation, and, in a small clinical trial, astaxanthin was shown to reduce the oxidation of low-density lipoprotein (LDL) and consequently lowered the risk of developing atherosclerosis (Iwamoto et al. 2000). Oxidative stress is often interlinked with inflammation, as inflammatory cells can lead to the release of reactive oxygen species (Hussain, Hofseth & Harris 2003) and reactive oxygen species can enhance proinflammatory gene expression (Flohé et al. 1997). Furthermore, 8 weeks of 8 mg/day astaxanthin reduced blood pressure and serum

lipids in patients with type 2 diabetes potentially due to protecting against glucose toxicity (Mashhadi et al. 2018).

Astaxanthin has potential for therapeutic use in chronic diseases, such as obesity, that are recognised to have dysregulated inflammation and oxidative stress (Fernández-Sánchez et al. 2011). Obesity is also associated with an increased risk of hypertension, hyperlipidaemia and insulin resistance and these have all been previously ameliorated by astaxanthin treatment (Choi, Youn & Shin 2011; Mashhadi et al. 2018). Furthermore, astaxanthin successfully reduced body weight gain in mice (Bhuvaneswari et al. 2010). Despite this, astaxanthin has been unable to reduce body weight in humans at a dose ranging from 6 – 20 mg/day tested in multiple clinical trials that were undertaken for up to 3 months (Xia et al. 2020). It remains to be seen why this health benefit was not translated from mice to humans, although it may potentially be due to the low bioavailability of astaxanthin.

It is well-recognised that astaxanthin has low bioavailability that was increased 3.7 fold by administering astaxanthin with lipid-based formulations (Mercke Odeberg et al. 2003). This is also the case with fucoxanthin showing an increased anti-obesity effect after being provided in the diet with medium-chain triglycerides (MCT) (Maeda, Hosokawa, Sashima, Funayama, et al. 2007). Body weight reduction in mice was observed when astaxanthin was incorporated with oil (Bhuvaneswari et al. 2010) but astaxanthin was not provided with lipid-based formulations in clinical trials so this may have had a role in this unsuccessful translation to humans (Choi, Youn & Shin 2011; Mashhadi et al. 2018; Yoshida et al. 2010).

The present study used a 16-week rat model of diet-induced metabolic syndrome (Panchal, Poudyal, Iyer, Nazer, Alam, Diwan, Kauter, Sernia, Campbell, Ward, et al. 2011) to determine whether astaxanthin extracted from *H. pluvialis* can reverse obesity, cardiovascular damage or detrimental liver changes. In previous studies, astaxanthin improved cardiovascular health with a dose of 75 mg/kg body weight in rats (Monroy-Ruiz et al. 2011) and liver health with a dose of 40 mg/kg body weight in mice (Shen et al. 2014), so similar outcomes are expected in the current study. This study differs from previous research in that it used a mixture of astaxanthin and fatty acids both extracted from *H. pluvialis,* named as an astaxanthin oleoresin. This serves both the purpose of increasing the bioavailability and further utilising the *H. pluvialis*

biomass for high-value products. Previous research in humans has indicated that a dose of 12 mg/day (0.2mg/kg/day in a 60kg human) or greater produces superior anti-inflammatory effects and a greater elevation of HDL-cholesterol in comparison to a lower dose (Xia et al. 2020), and to test this hypothesis in rats a low dose of 0.6 and a high dose of 1.2 mg/kg body weight/day were used. These doses equate to approximately 6 mg and 12 mg/day in humans (Reagan-Shaw, Nihal & Ahmad 2008). My hypothesis for this study was that the high dose astaxanthin would reduce blood pressure, serum lipids and obesity in diet-induced metabolic syndrome. I predicted that the major mechanism of these effects would be through the antioxidant and anti-inflammatory action of astaxanthin. I predicted that the low dose astaxanthin will not attenuate metabolic syndrome.

5.2 Methods and Materials

The materials and methods have been described in section CHAPTER 2 - MATERIALS AND METHODS with the following modifications.

5.2.1 Astaxanthin production and analysis

Biomass of *H. pluvialis* was cultivated by PacificBio at a land-based aquaculture facility near Ayr, Queensland, Australia (19°29’S, 147°28E). An astaxanthin oleoresin was extracted from *H. pluvialis* by PacificBio using an extraction process that is protected as intellectual property. Analysis was conducted by PacificBio to determine the concentration of astaxanthin (and isomers), lipids, proteins, carbohydrates, heavy metals, vitamins and other components in the oleoresin. The astaxanthin oleoresin was stored at -20 °C and combined with MCT oil to reduce viscosity, improve solubility and bioavailability when combining astaxanthin with the high-carbohydrate, high-fat (HCHF) and corn starch (CS) diets. The MCT oil was 100% derived from coconut oil and consisted of caprylic acid 55-70%, capric acid 29-45 %, < 3% lauric acid and < 2% caproic acid. The astaxanthin-MCT mixture that was administered in the HCHF and CS diet contained 80% astaxanthin oleoresin and 20% MCT oil.

5.2.2 Diet regimes

Rats (n = 72, weighing 338 ± 0.4 g) were randomly distributed into six experimental groups (n = 12 rats/group) as shown in Table 27.

Table 27: Treatment groups diet regime

Treatment Group	0 – 8 Week Diet	8 – 16 Week Diet
C	CS	CS
CAL	CS	CS + 0.6 mg/kg body weight/day astaxanthin
CAH	CS	CS + 1.2 mg/kg body weight/day astaxanthin
H	HCHF	HCHF
HAL	HCHF	HCHF + 0.6 mg/kg body weight/day astaxanthin
HAH	HCHF	HCHF + 1.2 mg/kg body weight/day astaxanthin

The astaxanthin treatment diets were prepared by gently heating the astaxanthin-MCT mixture to 40 °C using a baby bottle warmer (Tommee Tippee, Mount Waverly, Victoria, Australia) to bring the mixture to a liquid form. The results of the astaxanthin-MCT analysis determined that the mixture contained 10% astaxanthin, and this was used to determine the weight of the astaxanthin-MCT mixture to add into the treatment diets to obtain the desired doses. The average daily food consumption of CS and HCHF rats observed in the ulvan study was also used in this calculation. Astaxanthin is an unstable compound, so every day the treatment food in each individual bowl was emptied completely and refilled. The prepared treatment diets were stored in an airtight container in the dark and were kept for no longer than three days to limit exposure to oxygen and light.

5.2.3 Gut microbiome

Analysis was conducted as described in section CHAPTER 2 - MATERIALS AND METHODS with the following modification. The analysis was completed by Dr Marwan Majzoub at the University of New South Wales instead of Dr Priscila Goncalves.

5.3 Results

5.3.1 Astaxanthin composition

The astaxanthin oleoresin was analysed to determine the biochemical composition (Table 28).

Table 28: Astaxanthin oleoresin analysis

Biochemical Composition		Oleoresin % dry weight
Astaxanthin		9.5
Moisture		2.5
Ash		0
Proteins		0
Carbohydrates		0.7
Total lipids		87.5
Total saturated fatty acids	23.9	
Total mono-unsaturated fatty acids	18.5	
Total poly-unsaturated fatty acids	49.2	
Saturated fatty acids		g/100 g
C16:0 Palmitic acid		22.1
C18:0 Stearic acid		1.3
C14:0 Myristic acid		0.4
Mono-unsaturated fatty acids		g/100 g
C18:1(n-9 or n-7) Oleic acid		18.3
C16:1(n-7) Palmitoleic acid		0.2
Poly-unsaturated fatty acids		g/100 g
Total omega 3 fatty acids		13.1
C20:5(n-3) Eicosapentaenoic		0.4
C18:3(n-3) alpha-Linolenic		12.6
Total omega 6 fatty acids		36.1
C18:2(n-6) Linoleic		33.1
C18:3(n-6) gamma-Linolenic		1.3
C20:4(n-6) Arachidonic		0.9
C20:2(n-6) Eicosadienoic		0.4
C20:3(n-6) Dihomo-γ-linolenic acid		0.4
Heavy metals		mg/kg
Cadmium (Cd)		< 0.01
Arsenic (As)		0.7
Nickel (Ni)		0.1
Lead (Pb)		0.01
Copper (Cu)		1.6
Mercury (Hg)		< 0.01
Astaxanthin isomers		% of astaxanthin
Trans astaxanthin		73.2%
9-cis astaxanthin		8.2%
13-cis astaxanthin		17.7%

5.3.2 Physiology and metabolic indicators

HCHF diet-fed rats consumed less food than CS diet-fed rats; however, they consumed on average 171 more kilojoules of energy per day than CS rats as the HCHF diet has an increased energy density. The measurements at 8 weeks confirmed that HCHF diet-fed rats had a higher body weight and fat mass compared to CS diet-fed rats (Figure 46 and Table 29). The difference in body weight between the two diets continued to increase in the following 8 weeks of the study. Astaxanthin at both low and high dose did not change body weight, nor did it affect food or water intake. A dose of 0.60 and 1.2 mg/kg body weight/day was the aim of this study, however due to the HAH rats consuming more food than expected, HAL and HAH rats received a dose of 0.57 and 1.37 mg/kg body weight/day respectively. This trend of HAH rats consuming more food and fructose water than expected occurred throughout the full 16 weeks of this study. For the first eight weeks, HAH consumed 21% more food and 26% more water than H rats and 13% more food and 28% more water than H rats after astaxanthin was introduced into the diet in the final 8 weeks. This led to the energy intake of HAH rats far exceeding both H and HAL rats. The CS diet has a lower energy density, and consequently rats consume more food on this diet than the HCHF diet, and this led to the CAL and CAH rats receiving a dose of 1.15 and 2.50 mg/kg body weight/day respectively.

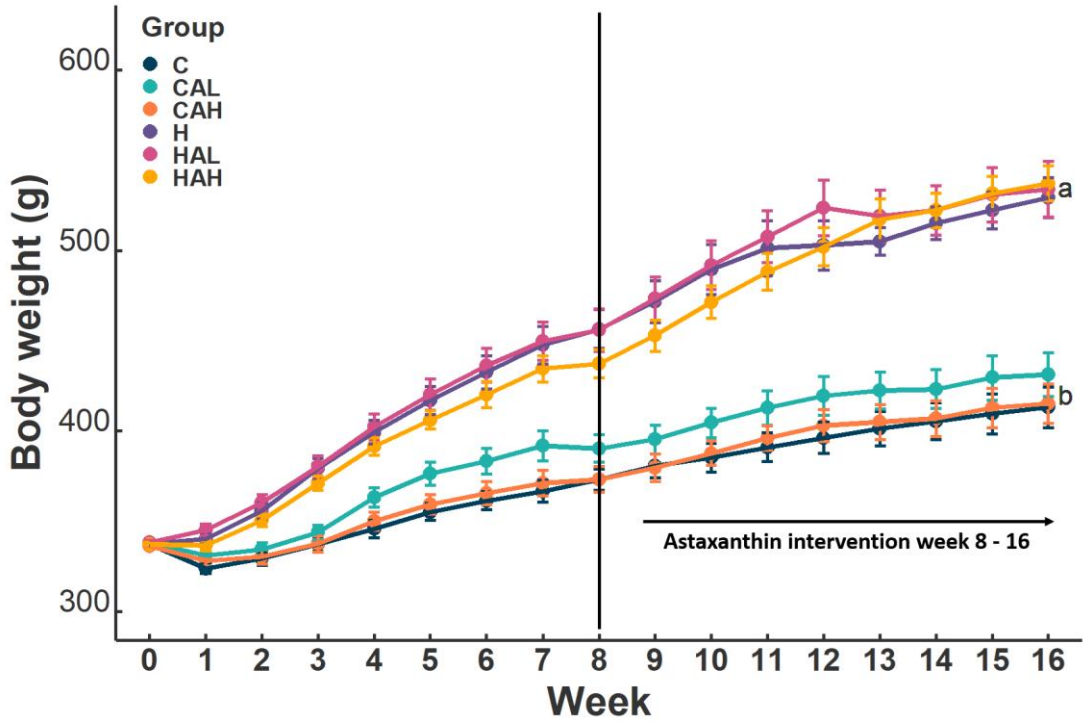


Figure 46: Body weight 0 – 16 weeks. Values are mean ± SEM, n = 12. Means without a common letter differ, p < 0.05. C, corn starch diet-fed rats; CAL, corn starch diet-fed rats supplemented with low dose astaxanthin; CAH, corn starch diet-fed rats supplemented with high dose astaxanthin; H, high-carbohydrate, high-fat diet-fed rats; HAL, high-carbohydrate, high-fat diet-fed rats supplemented with low dose astaxanthin; HAH, high-carbohydrate, high-fat diet-fed rats supplemented with high dose astaxanthin.

After 8 weeks, HCHF diet-fed rats had developed a reduced tolerance to an oral glucose load compared to CS rats (Table 29). The gap between HCHF diet-fed rats and CS-diet fed rats continued to grow from week 8 to week 16, but there was no significant effect of astaxanthin. Systolic blood pressure was measured at both 8 and 16 weeks, and there was a significant diet difference at both week 8 and week 16 (Table 29). There was no effect of astaxanthin treatment.

Table 29: Physiological parameters of rats fed control, low dose and high dose astaxanthin.

Variable	C	CAL	CAH	H	HAL	HAH	Diet	Treatment	Interaction
Body weight (g) week 8	373 ± 6^{b}	390 ± 8^{b}	373 ± 7^{b}	457 ± 11^{a}	456 ± 12^{a}	437 ± 8^{a}	< 0.001	0.139	0.480
Body weight (g) week 16	413 ± 11^{b}	431 ± 12^{b}	415 ± 11^{b}	558 ± 22^{a}	563 ± 19^{a}	546 ± 13^{a}	< 0.001	0.539	0.880
Body weight gain %	10.6 ± 1.8^{b}	10.3 ± 1.3^{b}	11.2 ± 1.7^{b}	21.9 ± 2.0^{a}	23.4 ± 1.1^{a}	24.9 ± 1.4^{a}	< 0.001	0.503	0.737
Water intake (g/day) week 0-8	35.0 ± 2.2^{a}	32.4 ± 1.8^{ab}	32.1 ± 1.9^{ab}	27.6 ± 1.1^{b}	29.8 ± 1.1^{ab}	34.8 ± 1.1^{a}	0.068	0.239	0.010
Water intake (g/day) week 8-16	24.9 ± 1.9^{b}	24.9 ± 1.5^{b}	24.4 ± 1.7^{b}	24.8 ± 0.9^{b}	27.8 ± 0.9^{ab}	31.7 ± 0.6^{a}	0.002	0.052	0.024
Food intake (g/day) week 0-8	42.2 ± 0.6^{a}	42.8 ± 0.7^{a}	42.5 ± 0.8^{a}	25.6 ± 0.9^{c}	27.9 ± 0.7^{bc}	31.1 ± 1.6^{b}	< 0.001	0.010	0.029
Food intake (g/day) week 8-16	40.5 ± 0.5^{a}	39.0 ± 0.8^{a}	40.9 ± 0.9^{a}	24.5 ± 0.7^{c}	23.8 ± 0.5^{c}	27.6 ± 0.9^{b}	< 0.001	0.001	0.178
Energy intake (kJ/day) week 0-8	473 ± 7^{c}	481 ± 8^{c}	478 ± 9^{c}	565 ± 18^{b}	612 ± 15^{b}	688 ± 28^{a}	< 0.001	< 0.001	0.002
Energy intake (kJ/day) week 8-16	452 ± 7^{c}	438 ± 9^{c}	459 ± 11^{c}	533 ± 15^{b}	532 ± 11^{b}	614 ± 17^{a}	< 0.001	< 0.001	0.007
Feed efficiency week 0-8	0.08 ± 0.01^{b}	0.11 ± 0.02^{b}	0.08 ± 0.01^{b}	0.21 ± 0.02^{a}	0.19 ± 0.02^{a}	0.15 ± 0.01^{a}	< 0.001	0.042	0.114
Feed efficiency week 8-16	0.09 ± 0.01^{b}	0.09 ± 0.01^{b}	0.09 ± 0.01^{b}	0.19 ± 0.02^{a}	0.19 ± 0.01^{a}	0.18 ± 0.01^{a}	< 0.001	0.664	0.720
Astaxanthin intake (mg/kg body weight/day)	0.00 ± 0.00^{d}	1.15 ± 0.04^{c}	2.50 ± 0.07^{a}	0.00 ± 0.00^{d}	0.57 ± 0.02^{c}	1.37 ± 0.05^{b}	< 0.001	< 0.001	< 0.001
Systolic blood pressure week 8 (mm Hg)	125 ± 3^{ab}	123 ± 2^{b}	129 ± 3^{ab}	137 ± 4^{a}	135 ± 4^{ab}	138 ± 4^{a}	< 0.001	0.561	0.885
Systolic blood pressure week 16 (mm Hg)	131 ± 4	135 ± 3	131 ± 4	146 ± 5	138 ± 5	139 ± 4	0.025	0.761	0.425
Fat mass week 8 (g)	63 ± 7^{b}	65 ± 5^{b}	59 ± 7^{b}	146 ± 18^{a}	126 ± 16^{a}	113 ± 12^{a}	< 0.001	0.345	0.475
Fat mass week 16 (g)	91 ± 7^{b}	108 ± 17^{b}	91 ± 15^{b}	219 ± 33^{a}	227 ± 25^{a}	201 ± 17^{a}	< 0.001	0.498	0.909

Table 30: Physiological parameters of rats fed control, low dose and high dose astaxanthin continued.

Variable	C	CAL	CAH	H	HAL	HAH	Diet	Treatment	Interaction
Lean mass week 8 (g)	293 ± 4	299 ± 5	303 ± 6	289 ± 7	317 ± 10	314 ± 7	0.070	0.061	0.314
Lean mass week 16 (g)	296 ± 4	303 ± 9	308 ± 9	305 ± 11	323 ± 10	328 ± 11	0.021	0.240	0.822
Tissue wet weight (mg/mm tibial length)									
Retroperitoneal fat	233 ± 23^{b}	305 ± 38^{b}	258 ± 33^{b}	616 ± 57^{a}	591 ± 63^{a}	604 ± 65^{a}	< 0.001	0.886	0.610
Omental fat	164 ± 16^{b}	192 ± 17^{b}	187 ± 14^{b}	342 ± 34^{a}	343 ± 30^{a}	336 ± 26^{a}	< 0.001	0.840	0.795
Epididymal fat	113 ± 14^{b}	135 ± 18^{b}	130 ± 13^{b}	249 ± 30^{a}	293 ± 37^{a}	254 ± 24^{a}	< 0.001	0.390	0.792
Total abdominal fat	507 ± 55^{b}	632 ± 69^{b}	576 ± 53^{b}	1207 ± 112^{a}	1226 ± 125^{a}	1194 ± 106^{a}	< 0.001	0.693	0.798
Kidneys	51 ± 1^{bc}	50 ± 3^{c}	49 ± 2^{c}	59 ± 2^{a}	58 ± 1^{ab}	59 ± 2^{a}	< 0.001	0.743	0.744
Liver	248 ± 11^{b}	256 ± 7^{b}	261 ± 6^{b}	378 ± 23^{a}	358 ± 11^{a}	375 ± 12^{a}	< 0.001	0.722	0.551
Left ventricle and septum	23.6 ± 0.7	23.9 ± 0.8	24.4 ± 1.1	25.2 ± 2.1	22.8 ± 0.6	24.7 ± 0.5	0.891	0.406	0.343
Right ventricle	4.7 ± 0.2	5.5 ± 0.3	5.6 ± 0.3	5.9 ± 0.5	5.8 ± 0.2	5.6 ± 0.3	0.039	0.351	0.183
Serum parameters									
ALT (U/L)	29.6 ± 4.6	23.9 ± 1.7	26.8 ± 2.0	30.2 ± 2.3	28.7 ± 1.8	29.7 ± 2.4	0.234	0.429	0.752
AST (U/L)	143.1 ± 8.8^{a}	92.9 ± 11.8^{b}	97.5 ± 11.0^{b}	131.4 ± 11.8^{ab}	100.6 ± 7.6^{b}	101.1 ± 8.0^{b}	0.879	< 0.001	0.588
Triglycerides (mmol/L)	0.65 ± 0.09^{cd}	0.56 ± 0.04^{d}	0.52 ± 0.05^{d}	1.75 ± 0.20^{a}	1.30 ± 0.15^{ab}	1.16 ± 0.16^{bc}	< 0.001	0.022	0.203
Total cholesterol (mmol/L)	1.64 ± 0.08	1.66 ± 0.07	1.66 ± 0.09	1.67 ± 0.07	1.74 ± 0.04	1.70 ± 0.07^{bc}	0.409	0.799	0.930
Oral glucose tolerance test									
AUC week 8 (mmol/L x min)	528 ± 17^{c}	552 ± 17^{bc}	544 ± 15^{bc}	604 ± 18^{ab}	618 ± 10^{a}	645 ± 20^{a}	< 0.001	0.217	0.554
AUC week 16 (mmol/L x min)	564 ± 26^{b}	566 ± 14^{b}	562 ± 15^{b}	648 ± 35^{ab}	704 ± 25^{a}	707 ± 30^{a}	< 0.001	0.423	0.429

Differing lower-case superscript letters indicate significant difference at $p<0.05$ determined by Tukey's HSD. ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the curve; C, corn starch diet-fed rats; CAL, corn starch diet-fed rats supplemented with low dose astaxanthin; CAH, corn starch diet-fed rats supplemented with high dose astaxanthin; H, high-carbohydrate, high-fat diet-fed rats; HAL, high-carbohydrate, high-fat diet-fed rats supplemented with low dose astaxanthin; HAH, high-carbohydrate, high-fat diet-fed rats supplemented with high dose astaxanthin.

5.3.3 Liver structure

The total wet liver weight was higher in HCHF diet-fed rats than CS diet-fed rats, but there was no change in astaxanthin treatment groups in comparison to controls (Table 25). Livers from H rats showed increased fat deposition causing hepatocyte ballooning compared to C rats (Figure 47). In the four rats per group that were analysed, the average score of liver fat deposition out of a maximum of 5 was 0.5 (C), 0.5 (CAL), 0.5 (CAH), 4 (H), 4 (HAL) and 4 (HAH). Hence, there was no change induced by astaxanthin. There was no difference in alanine aminotransferase (ALT) between H and C rats, but aspartate aminotransferase (AST) was increased in H rats in comparison to CAL and CAH (Table 25). Astaxanthin reduced AST in both CS and HCHF diet-fed rats.

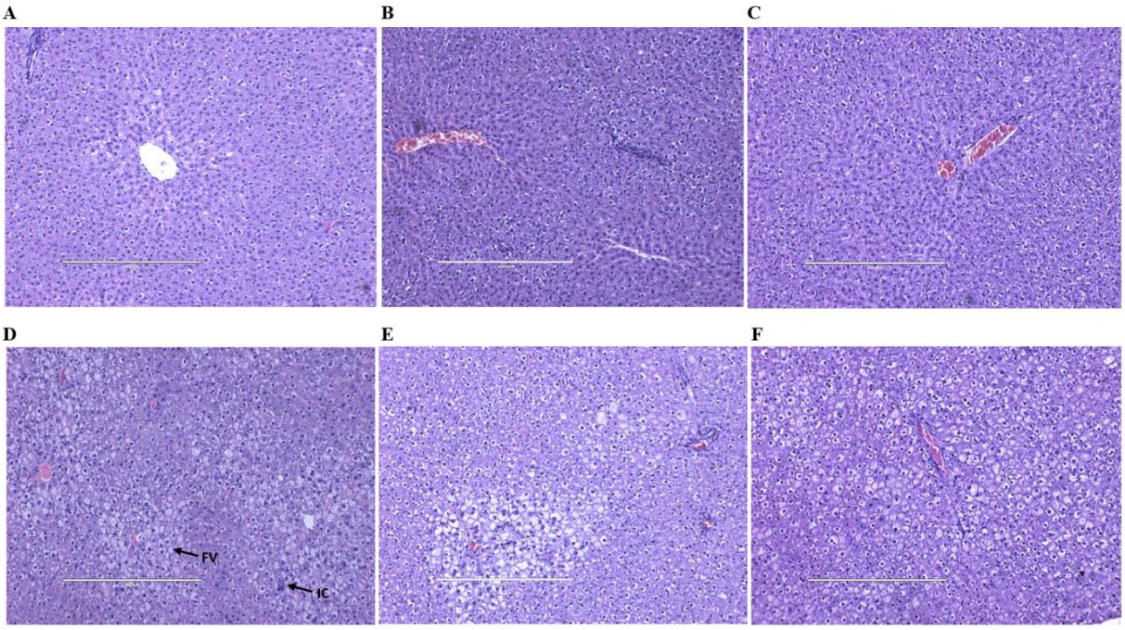


Figure 47: Haematoxylin and eosin staining of the liver to show fat deposition and inflammatory cell infiltration. Fat vacuoles are marked as 'FV' and inflammatory cells are marked as 'IC'. Scale = 400 µm. ***A*** *C liver.* ***B*** *CAL liver.* ***C*** *CAH liver.* ***D*** *H liver.* ***E*** *HAL liver.* ***F*** *HAH liver. C, corn starch diet-fed rats; CAL, corn starch diet-fed rats supplemented with low dose astaxanthin; CAH, corn starch diet-fed rats supplemented with high dose astaxanthin; H, high-carbohydrate, high-fat diet-fed rats; HAL, high-carbohydrate, high-fat diet-fed rats supplemented with low dose astaxanthin; HAH, high-carbohydrate, high-fat diet-fed rats supplemented with high dose astaxanthin.*

5.3.4 Gut microbiome

The gut microbiome was different between C samples and H samples without astaxanthin ($p = 0.017$), indicating an effect of diet on the bacterial community structure (Figure 48A). There was also a statistically significant effect of the addition of high dose astaxanthin to the HCHF diet ($p = 0.0027$), but not for the CS diet. There was no statistical support for differences in Shannon's diversity and richness due to either diet or astaxanthin supplementation (Figure 48B and 48C).

The most abundant bacterial classes found in the faecal samples for different treatment groups belonged to the classes Bacteroidia, Bacilli, Clostridia, Erysipelotrichia and Verrucomicrobiae (Figure 49A). An increase in the relative abundance of bacteria from the class Bacteroidia was observed in the C samples compared to the H samples. In contrast, there was an increase in the relative abundance of bacteria from the class Bacilli and Clostridia in the HCHF-diet fed rats compared to the CS diet-fed rats. High dose astaxanthin reduced the prevalence of Verrucomicrobiae in HCHF diet-fed rats.

An increase in the relative abundance of bacteria from the families *Muribaculaceae*, *Prevotellaceae* and *Erysipelotrichaceae* was observed in the CS diet-fed rats compared to the HCHF diet-fed rats (Figure 49B). In contrast, an increase in the relative abundance of bacteria from the families *Lactobacillaeceae* and *Lachnospiraceae* was observed in the HCHF diet-fed rats compared to the CS diet-fed rats. Astaxanthin reduced the abundance of *Akkermansiaceae*, *Lactobacillaceae, Muribaculaceae* and increased the prevalence of *Lachnospiraceae.*

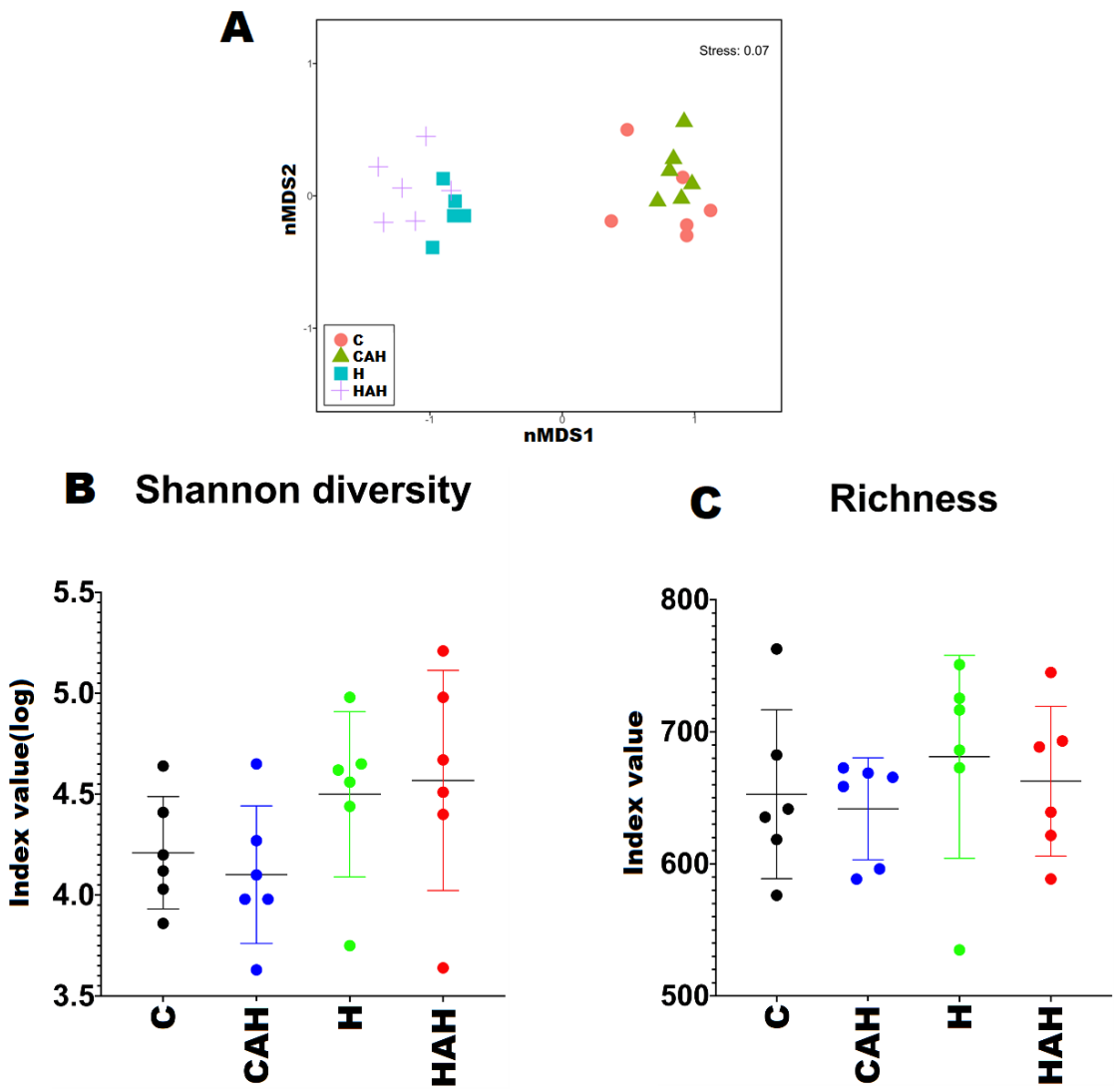


Figure 48: Effect of dietary supplementation of astaxanthin on the gut microbiota. ***A*** *Bacterial community structure of rat faeces under different diets. Non-metric multidimensional scaling (NMDS) plots incorporating all OTUs generated (n = 1190). In these plots, each point corresponds to one sample and incorporates the abundance of all OTUs present in that sample.* ***B*** *Shannon diversity of faecal samples.* ***C*** *Richness of faecal samples.*

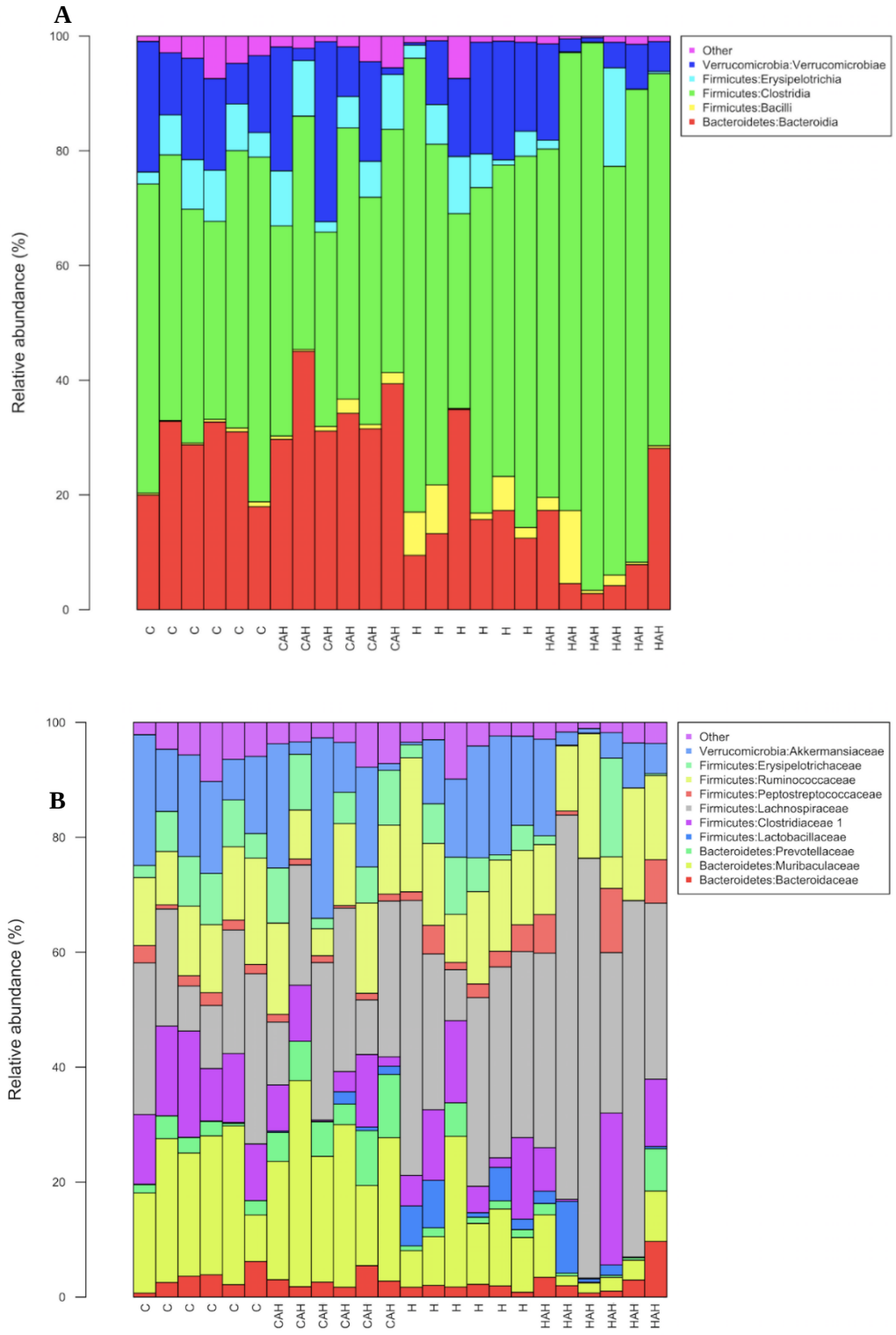


Figure 49: Taxonomic structure of the bacterial communities. ***A,*** *Taxonomic profiles of bacterial communities shown at the class level of all faecal samples.* ***B****, Taxonomic profiles of bacterial communities shown at the family level of all faecal samples.*

5.4 Discussion

In this study, the HCHF diet led to a phenotype associated with diet-induced metabolic syndrome including obesity, metabolic abnormalities, hypertension and ectopic fat deposition in the liver. This change was associated with a decrease in Bacteroidetes and an increase in the prevalence of Firmicutes in the gut microbiome. Introducing high dose astaxanthin into the HCHF-diet reduced serum triglycerides, but was unable to induce changes to body weight, deposition of fat in the liver, systolic blood pressure, body fat and glucose tolerance. The gut microbiome was impacted by high dose astaxanthin in the HCHF-diet, notably through reductions in the prevalence of *Akkermansia* spp (also reduced by CAH), *Muribaculaceae* spp., and increased prevalence of *Lachnospiraceae* spp. Low dose astaxanthin did not induce any changes to either CS diet-fed rats or HCHF diet-fed rats. The low dose of 0.6 and the high dose of 1.2 mg/kg body weight/day were selected based on the success of 6 mg and 12 mg/day astaxanthin doses in previous clinical trials (Xia et al. 2020).

Astaxanthin has been extensively tested in clinical trials at doses ranging from 5 to 12 mg/day (Donoso et al. 2021). Results include improvements to the serum lipid profile (Yoshida et al. 2010), oxidative stress (Choi, Youn & Shin 2011), skin health (Tominaga et al. 2017), cardiac function (Kato et al. 2020) and eye health (Saito et al. 2012) and reductions to systolic blood pressure and visceral fat (Mashhadi et al. 2018). These studies are supported by multiple other studies demonstrating a similar result with a comparable dose as shown in this large review of clinical trials by Donoso et al. (2021). In response to the success of astaxanthin clinical trials, astaxanthin is marketed in Australia and is allowed by the Therapeutic Goods Administration to make health indications of antioxidant, anti-inflammatory effects and the maintenance/support of skin health, cholesterol and healthy cardiovascular function (TGA 2019). Of the published clinical trials, there has been only limited studies showing non-significant effects of astaxanthin for these parameters. One clinical trial that tested a dose of 12 mg/day astaxanthin for 1 year in renal transplant patients found that astaxanthin had no effect on arterial stiffness, oxidative stress or inflammation (Coombes, Sharman & Fassett 2016).

With the success from most astaxanthin clinical trials, it was expected that the doses used in this current study would be effective because the low dose of 0.6 mg/kg body weight/day and high dose of 1.2 mg/kg body weight/day are comparable to 6 mg/day and 12 mg/day respectively for a 60 kg human (Reagan-Shaw, Nihal & Ahmad 2008). This calculation is based on the ratios of body surface area between rats and humans, and is a commonly used method for translating the results of an animal study into a clinical trial (Reagan-Shaw, Nihal & Ahmad 2008). However, the Reagan-Shaw method is self-admittedly not a precise measurement as it does not take into account pharmacokinetics or pharmacodynamics in the calculation, which can vary significantly between individuals and disease states (Vieira et al. 2019). This has led to criticism for use of this method for dose calculation, as there are concerns that inaccuracies could lead to underdosing and the consequent disregard of a potential treatment or overdosing and adverse effects (Blanchard & Smoliga 2015).

The other concern in using the Reagan-Shaw method for dose calculation is that it considers only body area, and not body composition (Pai 2012). This means that a patient who is 100 kg would receive double the dose of a patient weighing 50 kg, regardless of lean and fat mass proportions. This is of concern, considering that obesity is known to affect the volume of distribution of drugs (Hanley, Abernethy & Greenblatt 2010) and drug clearance (Pai 2010). Obesity is associated with oxidative stress, chronic low-grade inflammation and changes to the gut microbiome (Fernández-Sánchez et al. 2011; Tilg & Kaser 2011), and it is unknown whether these may affect pharmacokinetics as well.

In one study in mice, it was found that concentration of astaxanthin in the blood and liver, and accumulation in tissues and organs was greatly reduced in obese mice compared to mice of a normal body weight (Gao et al. 2020). Bioaccessibility in obese mice was reduced by 1/10, and this was speculated to be due to differences in lipid absorption between obese and lean phenotypes and the greater efficiency in freeing astaxanthin from esters observed in mice of a normal body weight (Gao et al. 2020). This is an example of why the dose should be first confirmed in an animal study using the appropriate disease state before converting that dose for a clinical trial. Another important comment is that the Reagan-Shaw method has been designed to calculate a human equivalent dose from

an efficacious animal dose (Reagan-Shaw, Nihal & Ahmad 2008). It may be more unreliable in calculating an animal dose from an effective human dose. This can be explored through examining the doses used in previous, successful animal studies testing astaxanthin.

There have been two clinical trials testing astaxanthin in obese humans, and these have observed antioxidant effects only in one study (Choi et al. 2011) and antioxidant, lowering of serum lipids and visceral fat reductions in the other (Mashhadi et al. 2018). Notably, body mass index and total body fat mass were not changed while % visceral body fat was reduced by only 5.9% with a dose of 8 mg astaxanthin for 8 weeks (Mashhadi et al. 2018). Astaxanthin has successfully reduced body weight and body fat with a dose of 6 mg/kg body weight/day in mice (Bhuvaneswari et al. 2010; Ikeuchi et al. 2007). When converting from mice to rats, the dose is halved, and this would mean this dose in mice would roughly equate to 3 mg/kg body weight/day in rats (Reagan-Shaw, Nihal & Ahmad 2008). However, this cannot be confirmed as this dose has not been previously tested in rats. Instead, when higher doses of 25 mg, 50 mg and 100 mg/kg body weight were tested in rats being fed a high sucrose diet over a period of 8 months, there was no change to body weight (Preuss et al. 2011). Similarly, a dose of 86 mg/kg body weight/day in rats was unable to reduce body weight and abdominal fat after a 6 week study (Kimura et al. 2014). It should be noted that in both of these rat studies, astaxanthin was provided in the diet in a powder form without being mixed with a lipid-based formulation, whereas, in the studies by Bhuvaneswari et al. (2010) and Ikeuchi et al. (2007), astaxanthin was combined with olive oil and administered directly through daily oral gavage.

Determining whether body weight could be reduced in rats by enhancing the bioavailability and stability of astaxanthin through combining it with lipids extracted from *H. pluvialis* was an aim of this study. However, the problems identified earlier with using the Reagan-Shaw formula for dose conversion may have resulted in the use of an unnecessarily low dose and consequently only a reduction in serum triglycerides was observed. Another possibility for the result of the current study is that the instability of astaxanthin may necessitate oral gavage rather than being provided in the diet as this would avoid prolonged exposure of astaxanthin to oxygen and light. This should be further

explored in future studies using a comparison between astaxanthin delivered through oral gavage and astaxanthin incorporated into the diet.

An astaxanthin dose formulation without lipid of 75 mg/kg body weight/day administered in the diet for 2 months was able to reduce blood pressure in Spontaneously Hypertensive Rats (Monroy-Ruiz et al. 2011). This blood pressure lowering effect was observed with an astaxanthin dose of 50 mg/kg body weight/day incorporated into the diet for 8 months in another study using a high sucrose model in rats to induce hypertension (Preuss et al. 2011). This dose of 50 mg in rats is equal to a dose of 486 mg for a 60 kg human, and this far exceeds the 8 mg dose required to lower blood pressure in type 2 diabetic patients (Mashhadi et al. 2018). Astaxanthin is expensive to produce, with a cost of $718 per kg, and the capsules available for purchase by consumers either come in 6 mg or 12 mg pill sizes (Li, J et al. 2011). Furthermore, the highest dose previously tested in humans is 20 mg/day (Xia et al. 2020) and consequently there is the danger of adverse effects from this considerably higher dose of 486 mg/day.

In the study by Preuss et al. (2011), a lower dose of 25 mg/kg body weight did not significantly reduce blood pressure. This dose of 25 mg/kg body weight was far greater than the high dose astaxanthin in this current study of 1.4 mg/kg body weight/day (HAH rats). The lipids incorporated to increase the bioavailability and stability of astaxanthin in this current study were not able to overcome this dose difference and induce an effect on blood pressure. Interestingly, this current study observed a reduction to serum triglycerides in HAH rats and these previous studies in rats did not despite testing this parameter. This effect of astaxanthin reducing triglycerides was observed in one clinical trial at a dose of 12 mg/day (Yoshida et al. 2010) and in the study in mice by Bhuvaneswari et al. (2010).

Astaxanthin has previously reduced fat deposition, inflammatory cell infiltration and fibrosis in the liver of both mice and rat studies (Bhuvaneswari et al. 2010; Kim et al. 2017; Shen et al. 2014). The dose used in rats was 40 mg/kg body weight/day without lipid formulation and this led to a reduction of ALT, AST and collagen deposition but fat was not examined (Shen et al. 2014). Shen et al. (2014) found that a 20 mg/kg/day dose did not produce any changes compared to control. The study in mice by Bhuvaneswari et

al. (2010) that used a dose of 6 mg/kg body weight combined with olive oil reduced liver weight, liver fat, inflammatory cell infiltration and necrosis in the liver. The other study that observed a positive effect of astaxanthin on liver health in mice used a dose of 0.015% astaxanthin in the diet, although unfortunately food intake was not measured so a comparison cannot be made (Kim et al. 2017).

In the present study, astaxanthin changed the gut microbiome. The Firmicutes/Bacteroidetes (F/B) ratio was not calculated for this study, however it would appear due to the reduction of Bacteroidetes and increase in Firmicutes in HAH rats compared to H, that the F/B ratio would be increased by high dose astaxanthin. The F/B ratio is recognised as biomarker for obesity in humans, as the F/B ratio is increased in obese people compared to lean people (Clemente et al. 2012). In the ulvan study previously tested during this PhD, the F/B ratio was reduced by ulvan supplementation and this correlated with a decreased body weight and fat mass gain. This suspected change in the F/B ratio was unexpected in this current study, as astaxanthin did not affect body weight or body fat.

However, it was observed that HAH rats did have altered food and water intake in comparison to H and HAL rats and it is recognised that diet can impact gut microbiome variation (Johnson et al. 2019). Alternatively, the altered gut microbiome may have been present prior to astaxanthin treatment and this could explain the increased food and fructose water intake. Future studies should ensure that the gut microbiome is analysed at baseline prior to treatment to ensure that any observed changes were not pre-existing.

The reduction observed to prevalence of Bacteroidetes appeared to be mostly due to the decreased incidence of *Muribaculaceae* spp. in HAH rats compared to H rats. A decreased prevalence of *Muribaculaceae* spp. was previously correlated with weight loss in obese mice (Ding et al. 2019; Park, S-E et al. 2020). Conversely, an increased prevalence of *Muribaculaceae* spp. was correlated with weight loss in obese mice in another study (Hou et al. 2020). This inconsistency in the correlations between physiological variables and the gut microbiome is not unique to *Muribaculaceae* spp., and is a recognised problem facing use of gut microbiome changes to predict and assess treatment responses (Biesiekierski, Jalanka & Staudacher 2019). Differences in study length, diet and

technique used to analyse the gut microbiome are examples of factors that commonly vary between studies, and these could potentially affect the gut microbiome. A greater understanding of the impact of these factors is required to improve consistency between studies.

A limitation of this study was the significant difference in food and water intake by HAH rats in comparison to H and HAL rats in the first 8 weeks of the study prior to treatment (*Table 25*). This trend continued for the final 8 weeks of the study. This culminated in HAH rats having a higher energy intake than H and HAL rats, and may have negatively impacted the chances of the high dose of astaxanthin in attenuating symptoms of metabolic syndrome. It is theorised that this may have occurred due to the treatment groups being grouped together on the cage racks rather than being separated, and also due to the treatment groups being decided at week 0 rather than week 8. It was tested and found that the temperature varied by 2 °C from the top row of the cage rack to the bottom row. To prevent this problem from occurring in future studies, rats from different treatment groups were arranged together in the cage racks one by one to minimise any possible effect of environmental factors. Furthermore, at the end of the 8-week experiments, rats were distributed into control and treatment groups to have as similar body weight and total fat mass totals as possible. This change was introduced for the remaining ulvan low dose and fucoxanthin studies of this thesis.

In conclusion, the high dose astaxanthin of 1.4 mg/kg body weight weight/day administered in the diet was unable to reverse symptoms of diet-induced metabolic syndrome, other than reducing triglycerides. This dose was selected based on the Reagan-Shaw calculation from a successful dose of 12 mg/day in humans to the equivalent dose in rats. The results of this study indicate that dose conversion between humans and rats is more complex than simply relying on body surface area differences, and this should be taken into account for future studies. Furthermore, an analysis of previous studies in mice and rats has demonstrated that combining astaxanthin with a lipid-based formulation such as olive oil, greatly improves response, even at a considerably lower dose. Despite the astaxanthin in this study being delivered in an oleoresin containing 88% lipids, this was unable to overcome the lack of responses to the low-dose astaxanthin.

CHAPTER 6 – CONCLUSIONS AND FUTURE RECOMMENDATIONS

6.1 Summary of findings

The objective of this research was to determine whether selected bioactive extracts from algae could prevent or attenuate symptoms of metabolic syndrome in an animal model. The bioactive compounds tested were ulvan, astaxanthin and fucoxanthin. The purpose of this research was to investigate in rats whether these compounds extracted from algae grown in tropical Queensland could be marketed as either a nutraceutical or functional food for humans. The results from animal studies are an important early step towards determining whether these algal extracts have therapeutic effects in humans. Further, the studies will help guide selection of an optimal dose for humans that is both efficacious and safe. In the animal studies conducted, both ulvan and fucoxanthin showed potential for the treatment of symptoms of metabolic syndrome. The research was undertaken in rats that developed many of the signs of metabolic syndrome by consuming a high-energy diet with increased simple sugars and saturated fats (HCHF diet) (Panchal, Poudyal, Iyer, Nazer, Alam, Diwan, Kauter, Sernia, Campbell, Ward, et al. 2011).

Ulvan intervention slowed the progression of obesity in HCHF diet-fed rats by lowering body weight gain and fat mass gain, and through decreasing overall fat deposition in the liver and omental fat pad compared to control. This anti-obesity effect was correlated with changes to the gut microbiome and reductions to feed efficiency. A key message from this study was that there was little benefit in quadrupling the dose of ulvan in rats from 0.25 g/kg body weight to 1 g/kg body weight. This lower dose equates to a dose of 4 g/day in an obese human weighing 100 kg. Obesity is linked to an increased risk of developing high blood pressure, type 2 diabetes and dyslipidaemia (Klop, Elte & Cabezas 2013; Tchernof & Després 2013; Yiannikouris et al. 2012). Consequently, by targeting obesity, ulvan is likely to reduce the incidence of these complications over the long-term even though no change to these complications of obesity were observed in this study.

Fucoxanthin intervention normalised systolic blood pressure and reduced fat deposition in the liver. The higher fucoxanthin dose decreased serum triglycerides but increased both low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol concentrations. Whilst increasing HDL-cholesterol and decreasing triglycerides is cardioprotective (Asia Pacific Cohort Studies Collaboration 2004; Després et al. 2000), elevated LDL-cholesterol concentrations heighten the risk of atherosclerosis, heart attack and ischaemic stroke (Cholesterol Treatment Trialists Collaboration 2010). This change was observed with a fucoxanthin dose of 7.5 mg/kg body weight/day, and this same effect has been previously observed in mice with a dose of 500 mg/kg body weight/day (Beppu et al. 2012). It appears that fucoxanthin upregulates cholesterol synthesis and lowers uptake and removal of LDL-cholesterol by the liver (Beppu et al. 2012).

In this thesis, astaxanthin at a dose of 1.4 mg/kg body weight/day lowered serum triglycerides in HCHF diet-fed rats. This dose was selected because it approximately equates to a dose of 12 mg/day for a 60 kg human, and findings from clinical trials have indicated that this dose produces health benefits in humans (Xia et al. 2020). Neither this dose, or a lower dose of 0.6 mg/kg body weight, were able to alter body weight, body fat, systolic blood pressure, glucose tolerance or fat deposition in the liver. Astaxanthin has previously attenuated these parameters in mice (Bhuvaneswari et al. 2010) at a dose of 6 mg/kg body weight/day and reduced blood pressure in rats with a dose of 50 mg/kg body weight/day (Preuss et al. 2011). It was concluded that the comparatively low doses used in this thesis were insufficient to attenuate metabolic syndrome in rats, and future studies should use a larger dose mixed with a lipid-based formulation to improve bioavailability.

A recurring observation when comparing the results of the studies of this PhD to previous studies was that many of the health improvements seen in prevention studies were not changed in a therapeutic intervention model. This was particularly evident when comparing the fucoxanthin study in this thesis to previous studies which were all prevention models where fucoxanthin treatment was given from the start of the study along with obesity-inducing diets. This trend held true even when the dose used in the prevention study was lower than the dose used in this thesis (Grasa-López et al. 2016). Obesity reduced bioaccessibility of astaxanthin in mice (Gao et al. 2020) and in humans

changed drug pharmacokinetics in general (Hanley, Abernethy & Greenblatt 2010; Pai 2010). While lifestyle changes are important in preventing obesity (Lavie et al. 2018), anecdotally, most drug treatments for obesity are given to already obese humans as reversal protocols. Consequently, testing potential drug treatments as interventions in obese rats rather than in lean, healthy rats is a more realistic challenge before attempting to translate results to clinical trials.

6.2 Implications of findings

The findings of this thesis advocate the use of ulvan in the treatment of metabolic syndrome; however, this has yet to be demonstrated in humans. Ulvan contributes between 9 – 36% of the dry weight of the genus *Ulva*, which is an edible, green sea-lettuce ubiquitous worldwide (Kidgell et al. 2019). Taking the average of this ulvan content in *Ulva*, this would mean that a human weighing 100 kg would need to consume 18 g of dried *Ulva* to obtain the human equivalent dose of 4g/day ulvan (Reagan-Shaw, Nihal & Ahmad 2008). This is feasible as a component of foods and supports the consumption of *Ulva* for treating obesity, and through consuming the whole biomass there is the possibility of further health benefits from other algal components. *Ulva* contains angiotensin 1-converting enzyme inhibitory peptides that could potentially be used to lower an increased blood pressure (Paiva et al. 2016; Sun et al. 2019). Furthermore, *Ulva* contains other important nutrients, for example dried *Ulva lactuca* collected in Indonesia contained 28% dietary fibre, 14% protein, 1828 mg/100g calcium and 467 mg/100 g potassium (Rasyid 2017).

Fucoxanthin is the major carotenoid produced in brown macroalgae, for example in two species commonly consumed in Japan called *Undaria pinnatifida* (used in soups and salads) and *Laminaria japonica* (used in soups) (Zhang et al. 2015). The content of fucoxanthin in brown macroalgae varies in response to geographical location and time of harvesting (Fung, Hamid & Lu 2013), however *U. pinnatifida* has been previously reported to contain 0.98 mg fucoxanthin/g dry weight (Grasa-López et al. 2016). The low dose fucoxanthin used in this thesis was 0.8 mg/kg body weight/day, and this approximately equates to a dose of 10 mg/day in a human weighing 100 kg (Reagan-Shaw,

Nihal & Ahmad 2008). Consequently, it is feasible to consume 10 g of dried *U. pinnatifida* as a component of food to obtain a sufficient fucoxanthin dose to reduce systolic blood pressure. Furthermore, previous comparisons between commercial dried *U. pinnatifida* and the fresh seaweed have suggested that fucoxanthin may be more stable with the other organic components in the seaweed rather than being extracted and isolated (Mori et al. 2004). *U. pinnatifida* and other brown seaweeds have the added bonus of containing the sulphated polysaccharide fucoidan (Zhao, Y et al. 2018). Similar to ulvan, fucoidan is a soluble fibre that has shown anti-obesity, anti-dyslipidaemia and anti-diabetic effects in animal studies with a dose of 200 mg/kg body weight/day (Kim, Jeon & Lee 2014; Shang, Song, et al. 2017). This equates to a dose of 1.6 g/day in a human weighing 100 kg (Reagan-Shaw, Nihal & Ahmad 2008). *U. pinnatifida* contains a variable amount of fucoidan depending on time of year, temperature, nutrient concentration and reproductive activity of the plant. However, the fucoidan content can reach 16% dry weight (Skriptsova et al. 2010). Therefore, a human would need to consume 10 g of dried *U. pinnatifida* to obtain roughly a 1.6 g dose of fucoidan.

The discussion above of the bioactive compounds for *Ulva* and *U. pinnatifida* is limited by the current understanding of nutritional composition across different algal species, locations, and seasons. There may be other algae that are currently less studied that could possess other unique compounds or promising combinations of bioactive compounds. For example, a recent study examined 26 tropical macroalgae species from French Polynesia and focussed on those that had a lack of previous studies (Zubia et al. 2020). This study found that multiple Polynesian species such as *Dictyota hamifera*, *Chnoospora minima* and *Amansia rhodantha* showed potent antioxidant, anti-diabetic and anti-cancer effects and yet there has been very limited research into these seaweeds. When Zubia et al. (2020) analysed the bioactive compounds from *A. rhodantha*, two antioxidant compounds not previously reported in the marine environment were found, however further work is needed to understand the structure and source of these compounds. This emphasises that further characterisation of a diverse range of algae is needed to allow new discoveries of potential bioactive components. In particular, the potential applications of algal bioactive peptides for treatment of high blood pressure, oxidative stress, cancer, atherosclerosis and osteoporosis are only just starting to be understood (Fan et al. 2014). It is recommended

that these newly discovered bioactive compounds should be tested individually to confirm that they are responsible for the observed health benefits and to determine an optimal dose.

6.3 Challenges facing the therapeutic use of algae

Research to understand the therapeutic effects of algae is not a meaningful contribution unless the algae sample is analysed to determine the composition. This is because of the large variability in the biomass composition in response to environmental conditions such as season, light intensity, temperature and salinity (Metsoviti et al. 2019). This variability can lead to large differences in nutrient composition, for example the phenolic content of *Euglena gracilis* varied from 137 μg g^{-1} in one study (Cervantes-Garcia et al. 2013) to 5800 μg g^{-1} in another study due to differences in cultivation technique and potentially strain (Metsoviti et al. 2019). Phenolic compounds are potent antioxidants and this activity could lead to greatly varying therapeutic effects in response to higher than average or lower than average values. This could consequently oversell the potential of an algal species or result in that algal species being disregarded unnecessarily from future research. Further research is required to analyse high-performing strains and improved cultivation techniques to produce a more desirable nutrient composition.

Additionally, there are challenges facing use of algae in Australia as there is limited knowledge about how the Australian climate affects algal growth and composition, as well as the range of endemic species. The large diversity of the *Ulva* genus will be used to emphasise this point. Approximately 400 different *Ulva* species have been reported to the AlgaeBase database, and of these 84 species are accepted taxonomically (Guiry & Guiry 2021). However, when 53 *Ulva* samples were collected from sites across southern Australia in 2010, it was found that only half of the total species were a match for the documented *Ulva* species and the remaining were unique species (Kraft, Kraft & Waller 2010). This highlights that the Australian *Ulva* flora is comprised of species that are not commonly found in other regions, and further work to collect and characterise these and other unique Australian algae is required. It is important to have a thorough understanding of local Australian algae, as previous work has demonstrated that certain species and

strains of *Ulva* have improved growth rates in conditions matching the local climate in comparison to others (Lawton et al. 2013).

Australia is otherwise well-suited to an expanded algal industry due to the availability of coastline and non-arable land that is not suitable for traditional crops. Furthermore, algae have been successfully grown in a range of wastewater from sewage, manufacturing industries, aquaculture and agriculture (Arumugam et al. 2018). This could complement the growing aquaculture and agriculture industry in Australia (Department of Agriculture 2020), and has already been implemented by companies such as Pacific Bio (https://www.pacificbio.com.au/) in North Queensland. When expanding the algal industry in Australia, it is important to learn from the mistakes made in algae farming in Southeast Asia, which included loss of genetic variability in farmed strains, bioinvasion of introduced seaweeds and overuse of non-environmentally friendly practices (Hurtado, Neish & Critchley 2019). Other actions that need to be taken include publication of a comprehensive review of Australian algae species, investigation of the suitability of additional regional areas for algal industry development, and at later stages, further testing of selected species to determine optimal growing conditions and strain.

However, expansion of the algal industry will always be hindered unless the Australian general population becomes willing to consume seaweed and seaweed products. Australian interest in seaweed has been growing in recent years due to the rising popularity of seaweed products such as sushi and the recognition of its nutritional quality (Birch, Skallerud & Paul 2018). There are still barriers to public acceptance of seaweed including apprehension about how to prepare, use and store seaweed and dislike of the smell, texture and taste (Birch, Skallerud & Paul 2018). These could be overcome with public education or instructions on seaweed packaging to help consumers to try something out of their comfort zone, for example by public broadcasters such SBS (https://www.sbs.com.au/food/ingredient/seaweed) or incorporation of seaweed into other commonly consumed foods such as pizza (Combet et al. 2014). Alternatively, functional food products could be developed by mixing just the extracted bioactives from algae, such as ulvan, within other food products. This could potentially deliver health benefits without imparting the strong ‘seaweed’ taste that is undesirable to some consumers. Furthermore,

incorporating seaweed into food products could result in other benefits such as antimicrobial and antioxidant effects of seaweed added to beef patties (Cox & Abu-Ghannam 2013). Positive results from large clinical trials showing the health benefits of seaweed would also greatly help adoption of seaweed into the Australian diet.

6.4 Limitations and recommendations

There were limitations to the research conducted in this thesis. A major limitation is that the mechanisms behind the majority of the physiological changes observed could only be theorised and were not able to be explored further. Molecular biology studies on liver samples were underway when the research laboratory was closed in March 2020 as part of the USQ response to the coronavirus pandemic. As a different approach, an attempt was made in the ulvan studies to undertake an in-depth correlation of physiological responses with changes in the gut microbiome. However, the changes observed resulted in further questions on why the gut microbiome was altered and whether this was a result of the treatment or an indirect effect from another change. Short-chain fatty acids were unchanged by ulvan treatment, and so the mechanism by which ulvan affected feed efficiency and the gut microbiome remains unknown. Future testing to analyse the lipid and bile acid content of faeces to determine whether the absorption of nutrients is altered is recommended for future studies.

The mechanisms behind the blood pressure lowering effect of fucoxanthin were also not explored. It was theorised that the antioxidant effect of fucoxanthin may have contributed to this effect and this could be further explored by analysing serum concentrations of antioxidant enzymes such as superoxide dismutase or glutathione peroxidase. Molecular work to quantify expression of genes such as sterol regulatory element-binding protein 1 and 2 would confirm that fucoxanthin can increase cholesterol synthesis and decrease liver cholesterol clearance as shown in previous studies. It would be interesting to examine whether these genes have also been changed in response to the lower dose of fucoxanthin, as this would suggest that in the long-term LDL-cholesterol concentrations would still increase. Analysing serum concentrations of fucoxanthin and astaxanthin metabolites in response to feeding these treatments in the diet is another important recommendation to

confirm that rats are obtaining the expected dose as the reported oral bioavailability of these compounds is low.

There were several limitations to the study design in this thesis. Grading of the extent of fat deposition in the liver was determined visually using a light microscope and this allows for human error and potentially bias. Greater accuracy and prevention of bias can be achieved by collecting liver samples and quantifying triglyceride and cholesterol content using assay kits such as those produced by Abcam. Metabolic syndrome was also not completely induced in high-carbohydrate, high-fat (HCHF) diet-fed rats. In all studies of this thesis, the response to the glucose tolerance test was the same for both HCHF diet-fed rats and corn starch (CS) diet-fed rats. Consequently, the treatments were tested in rats that had normal glucose tolerance, and this is not a true test of whether these interventions have anti-diabetic effects. The HCHF diet used is also a fixed diet, unlike the majority of human diets, and is thus not a complete replica of the obesogenic human diet that this model is attempting to mimic. Only male rats were used in the studies of this thesis, and this is a limitation because the results observed are consequently not generalisable to females. Males were used as most of the literature studies used for comparison of these studies were on male rats, and future studies should use female rats to confirm the results seen.

The gut microbiome was analysed at the completion of the 16-week period and this measurement was used to describe the effects of the treatment. At this timepoint, there was major variability between rats within the same treatment group and it was at times difficult to observe trends in the data due to this. Analysing the gut microbiome at the 8-week timepoint prior to treatment would allow for an understanding of the starting point and whether there is already gut microbiome variation. Consequently, the 8-week values could be directly compared to the 16-week values and this change for each rat could be analysed rather than the average across the group. It is recognised that old age is associated with decreased gut microbiome diversity, and this promotes increased risk of inflammation and disease (Ragonnaud & Biragyn 2021). There is further concern that this may impact nutrient absorption, and this has been observed with the effects of ageing on protein digestion and absorption (Watson, Cross & Grosicki 2021). This should be taken

into consideration when attempting to translate the results seen in this thesis to an aged population.

If possible, it is also recommended to determine the gut microbiome species rather than stopping at the genus level as occurred in this thesis. This was performed in this thesis in response to the recommendations of the collaborator at the University of New South Wales due to the integrity of the 16S ribosomal RNA samples. Both ulvan and astaxanthin reduced the relative abundance of *Akkermansia* spp in this thesis, and it is important to understand which species has decreased as one is associated with increased fat mass and metabolic syndrome and the other is not (Ouwerkerk et al. 2016). This is just one example of why it is vital to understand the gut microbiome changes at the species level.

Another important observation from this thesis is that comparing two or more different dosages within the same study can highlight whether there is an unusual response to a higher or lower dose as well as the lowest effective dose. For example, fucoxanthin showed potential for improving cardiovascular health by reducing systolic blood pressure with both the lower and higher dose tested. The lower dose of 0.8 mg/kg body weight in rats appeared to be moving towards a reduced LDL-cholesterol concentration ($p > 0.05$), but the high dose of 7.5 mg/kg instead elevated LDL-cholesterol. This suggests that a dose somewhere in the middle could decrease LDL-cholesterol, as has been previously observed with a dose of 1 mg/kg body weight in rats (Grasa-López et al. 2016). It is critical that the dose-response range is understood for fucoxanthin, as it is not currently feasible to recommend fucoxanthin to a patient with poor cardiovascular health. This is because high concentrations of LDL-cholesterol increase the risk of worsening their condition, despite the blood pressure-lowering effect. The importance of comparing responses from multiple dosages within the same study has been observed previously in an ulvan study, where they found that the best response was provided with the intermediate dose from the three doses tested (Li et al. 2020). Comparing responses from different doses within the same study is more valuable than separate studies as there are often major differences in the design of the model used and potentially the structure of the algae extract used.

6.5 Conclusions

This thesis has provided novel insights into the knowledge gaps regarding these three algal extracts. This is the first study to examine the effects of ulvan on the gut microbiome *in vivo*, and the first to test both ulvan and fucoxanthin in a therapeutic intervention style model rather than prevention. The minimal response to astaxanthin in this thesis was unexpected, although it still provides dose guidance to future studies wishing to use a lipid-based formulation to improve astaxanthin bioavailability. The advantage of using animal studies to test these interventions is the ability to monitor histological, biochemical and tissue weight changes that are otherwise difficult to achieve in clinical studies. Furthermore, they allow complete control over the participant diet and prevention of other potential confounding variables. Randomised, double-blind placebo-controlled clinical trials are necessary as the next step to confirm whether the therapeutic effects observed in this thesis can be translated to humans. Fucoxanthin requires further animal studies to understand the optimal dose to induce both blood pressure lowering effects and fat reduction, whilst avoiding increased LDL-cholesterol concentrations.

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