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The effects of model foods rich in bioactive compounds on brain-gut regulation and neurodegeneration

A thesis
submitted in partial fulfilment
of the requirements for the Degree of
Doctor of Philosophy in Food Science

at
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by
A K M Mofasser Hossain

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By

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A compelling interaction between the diet, gut and host physiology has emerged. Biologically active compounds particularly dietary fibre (DF) and polyphenols in a diet have long been appreciated due to their inverse relation with rising chronic diseases, particularly metabolic disorder, obesity, type 2 diabetes and cancers. Research has illustrated a complex interaction of multiple peptides (gut endocrine systems) and nutritional inputs physiologically affect appetite control and host immunity. Despite the potential health benefits of consuming a diet rich in bioactive compounds, little is known about the bio-functional interactions such as digestibility, effects of digested and fermented metabolites on the attenuation of gut endocrine system and immunity of the host.

This study developed a cookie from wholemeal cereals which incorporated different proportions (0, 5, 10 and 15 %) of blackcurrant or astaxanthin-rich microalgae *Haematococcus pluvialis* powder as a replacement of wheat, barley and oat flours. The study evaluated the effects of blackcurrant/astaxanthin on cookie textural properties. The *in-vitro* carbohydrate digestibility of the cookies demonstrated the glycaemic control. Inclusion of blackcurrant/astaxanthin powder in wholemeal cereals cookies significantly ($p < 0.05$) decreased the rate of glucose release, when compared with wholemeal cereals cookie control. The 15 % blackcurrant and astaxanthin powder incorporated cookies had a significantly ($p < 0.05$) higher fibre fraction, compared to the control flour cookies. Blackcurrant powder contains about 25 % and astaxanthin-rich *H. pluvialis* powder contains more than 30 % DF.

Using an *in-vitro* fermentation model, the prominent gut bacterial growth and fermentative properties of the indigestible cookie fraction were evaluated. The combination of four bacterial species *Lactobacillus* and *Bifidobacterium* was utilised using the isolated fibre fractions from different cookies as substrates. These fibre fractions significantly increased the microbial growth and their metabolite (short-chain fatty acids, SCFAs) production. The blackcurrant/astaxanthin incorporated substrates significantly increased the bacterial populations, lowered fermentation pH and increased optical density (OD600) at 6, 12, 24 and 48 h incubation when compared with negative (medium only) and positive control (wholemeal cereals cookies). The increased concentrations of SCFAs production from the *in-vitro* bacterial fermentation indicated selective fermentation capacity of blackcurrant/astaxanthin cookie fibre fractions.

The simulated digestion and fermentation and cellular biological activity of the cookie digesta was studied and revealed possible links between metabolites and various physiological processes associated to health and diseases. Chemical extraction of free, bound and total phenolics of the blackcurrant/astaxanthin cookies exhibited significantly higher radical scavenging capacity in chemical and cell models, which is believed to have the inverse relationship with many chronic diseases such as cancer. The incorporation of blackcurrant/astaxanthin significantly ($p < 0.05$) increased the total phenolic content (by about 60 %), significantly improved oxygen radical absorbance capacity (by about 25 %) of the cookie. The higher ORAC and DPPH indicated that blackcurrant/astaxanthin incorporated cookie digesta/fermenta/extracts were strong antioxidants, which was in accordance with the higher total phenolic content. The major phenolic acids of the digesta/extracts were assessed by RP-HPLC, and were found to be ferulic acid, *p*-hydroxy benzoic acid and *p*-coumaric acid. However, quercetin-3-rutinoside, quercetin derivatives and kaemferol-3-glucoside were only found in blackcurrant incorporated cookie extracts. Whereas, the cinnamic and salicylic acid were found in astaxanthin incorporated cookie extracts.

Major therapeutic target of cancer diseases, the inhibition of abnormal cell growth and/or proliferation activity and cellular oxidation were observed when using the cookie digesta/fermenta/extract on HepG2 cell model. Importantly, qRT-PCR exhibited significantly higher

mRNA levels of the appetite regulatory cholecystinin (CCK), glucagon-like peptide-1(GLP-1), gastrin-releasing peptide (GRP) and nucleobindin-2 (NUCB2)/nesfatin genes (ten to twelve-folds) expression. This significant interplay in genetic modulation reflected the positive link between signalling molecules SCFAs and/or bioactive phenolics and appetite control. The lower levels of inflammatory cytokines interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and inflammation activator nuclear factor-kappa B (NF-kB) (half to two-fold) was demonstrated in HepG2 cells treated with blackcurrant/astaxanthin incorporated cookie. The anti-oxidative/inflammation response of digesta/fermenta/extract in dynamic biological changes could be a therapeutic alternative to facilitates metabolic homeostasis, counter inflammation and enhance immunity.

Metabolites from gut microbiota interact with the brain physiology through signalling molecules SCFAs are starting to emerge. Interestingly, our study demonstrated that the bacterial metabolites SCFAs did not significantly inhibit amyloid- β (A β) aggregation in a cell model of Alzheimer's disease.

Enhanced functionalities including glycaemic control, antioxidation, anti-proliferation and genetic modulation of the bioactive compounds in cookie accelerates our knowledge to regulate appetite, oxidative stress and inflammation. Noteworthy, the study of cell model reveals the possible links between food intake and the manipulation of brain and gut health. The exerted synergistic effects of this study suggest that there may be a new and effective option to prevent and control chronic diseases in human.

Keywords: glycaemic control, simulated digestion, bacterial fermentation, dietary fibre, free phenolics, bound phenolics, short-chain fatty acids, antioxidant-dietary fibre, human cancer cell-line HepG2, E22G-mCherry Hek 293 cell, cellular antioxidant analysis CAA, inflammatory markers, satiety, cytotoxicity, anti-proliferation, amyloid- β protein, Alzheimer's disease

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Abbreviations

A β -amyloid-beta

AD-Alzheimer's disease

Antioxidant-DF-antioxidant-dietary fibre

BBR-blood brain barrier

BCC-wholemeal barley flour cookie control

B5A-wholemeal barley flour with 5 % astaxanthin-rich microalgae *Haematococcus pluvialis* powder

B10A-wholemeal barley flour with 10 % astaxanthin-rich microalgae *Haematococcus pluvialis* powder

B15A-wholemeal barley with 15 % astaxanthin-rich microalgae *Haematococcus pluvialis* powder

B5B-wholemeal barley flour with 5 % blackcurrant powder

B10B-wholemeal barley flour with 10 % blackcurrant powder

B15B-wholemeal barley flour with 15 % blackcurrant powder

BMI-body mass index

CCK-cholecystokinin

CAA-cellular antioxidant activity

CNS-central nervous system

CVD-cardiovascular disease

DF-dietary fibre

DW-dry weight

GI-glycaemic index

GL-glycaemic load

GRP-gastrin-releasing peptide

GGE-glycaemic glucose equivalent

GLP-glucagon-like peptide

HepG2-human hepatocellular liver carcinoma

IDF-insoluble dietary fibre

IL-1 β -interleukin-1 β

IL-6-interleukin-6

MetS-metabolic syndrome

NF- κ B-nuclear factor-kappa B

NUCB2/nesfatin-nucleobindin-2 (NUCB2)/nesfatin

OCC-wholemeal oat flour cookie control

O5A-wholemeal oat flour with 5 % astaxanthin-rich microalgae *Haematococcus pluvialis* powder

O10A-wholemeal oat flour with 10 % astaxanthin-rich microalgae *Haematococcus pluvialis* powder

O15A-wholemeal oat flour with 15 % astaxanthin-rich microalgae *Haematococcus pluvialis* powder

O5B-wholemeal oat flour with 5 % blackcurrant powder

O10B-wholemeal oat flour with 10 % blackcurrant powder

O15B-wholemeal oat flour with 15 % blackcurrant powder

PA-phenolic acids

SCFA-short-chain fatty acids

SDF-soluble dietary fibre

T2DM-type 2 diabetes mellitus

WCC-wholemeal wheat flour cookie control

W5A-wholemeal wheat flour with 5 % astaxanthin-rich microalgae *Haematococcus pluvialis* powder

W10A-wholemeal wheat flour with 10 % astaxanthin-rich microalgae *Haematococcus pluvialis* powder

W15A-wholemeal wheat flour with 15 % astaxanthin-rich microalgae *Haematococcus pluvialis* powder

W5B-wholemeal wheat flour with 5 % blackcurrant powder

W10B-wholemeal wheat flour with 10 % blackcurrant powder

W15B-wholemeal wheat flour with 15 % blackcurrant powder

WHO-World Health Organization

Chapter 1

Introduction

Worldwide obesity has increased to epidemic proportions. Cardiovascular disease (CVD), type 2 diabetes mellitus (T2DM), metabolic disorder, neurodegeneration and certain forms of cancers are the major consequences of obesity. Comprehensive studies in diverse populations (race/ethnic, income, age and sex) have reported a continual rise in obesity throughout the world, and that obesity levels have nearly doubled since 1990 (WHO 2017). The leading causes of death, including cardiovascular diseases (No.1 cause of death globally), cancer (2nd) and diabetes (7th leading cause of death by 2030, 2.2 million deaths were contributed to high blood glucose), are the results of being overweight and obese (WHO 2017). In 2014 globally about 13 % of the population (11% men and 15% women) were obese and 39 % (38 % men and 40 % women) were overweight (WHO 2017). In addition, the prevalence of childhood obesity is high (41 million children under 5 years of age were overweight or obese in 2014) and nearly 90 % of children with obesity remain obese into their adult life (WHO 2017). The prevalence of and rising trend of, overweight and obesity varies in different populations. New Zealand has the third highest rate (about one-third of total population) of overweight and obese in the OECD (organization for economic cooperation and development) (OECD 2015; Utter et al. 2015). There have been substantial rises in overweight and obesity rates have increased remarkably (both adults and children) in New Zealand over the three decades (Utter et al. 2015). According to these projections and the acknowledgment of epidemiological studies, it is clear that the rapidly growing obesity and obesity-related comorbidities are associated with the worldwide clinical and public health burden.

Overweight and obesity has been described by the world health organisation (WHO) as - “abnormal or excessive fat accumulation that may impair health”; and can be assessed by the body mass index (BMI; kg/m^2) which is in scale ≥ 25 overweight and ≥ 30 obese (WHO 2017). Waist circumference (body fat distribution; waist : hip ratio) is also an important predictor to measure the morbidity (Sjostrom 1993). Although energy imbalance (excess energy intake than energy expenditure) is the

fundamental cause of obesity, the aetiology of obesity epidemic is complex and diverse including environmental (such as diet and physical activity), genetic and physiological that contribute in varying degree to promote the development of obesity (Heshka & Allison 2001; Greenway 2015). Nutritional transition (diet composition such as diet high in fat, simple carbohydrate and animal products and low in complex carbohydrate and fibre) is thought to be the primary cause of overweight and obesity (Swinburn et al. 2004). Diets are dependent on the different metabolic process by which diet produces a potential to biological mechanism (such as energy density, satiety and metabolic responses) after digestion. There is increasing evidence that higher carbohydrate intake, particularly of readily absorbed simple carbohydrates, is related to an increase in obesity and type 2 diabetes mellitus (T2DM) (McMillan-Price & Brand-Miller 2006). It has also been suggested that diet high in fat lead to weight gain because of its high energy density and lower impact on satiety (Hellström 2013; Attuquayefio et al. 2016). The physiological state of obesity is associated with alterations in hormones production/sensitivity (such as insulin, leptin and adiponectin) and metabolism, which may alter appetite control and relative substrate oxidation, such as carbohydrate versus fat (Van Dam & Seidell 2007; Attuquayefio et al. 2016).

Oxidative stress is one of the causes of the deterioration of cellular integrity, structure and chemical functions; and cellular response mechanisms have been implicated for the progression of major chronic diseases such as neurodegeneration (St-Pierre et al. 2006), aging (Haigis & Yankner 2010), obesity (McMurray et al. 2016), T2D (Tangvarasittichai 2015) and cancer (Panieri & Santoro 2016). Elevated levels of intracellular reactive oxygen species (ROS; including O_2^- , H_2O_2 and $\bullet OH$ free radical) are the primary cause of oxidative stress. Under normal biological phenomena, ROS acts as signalling molecules to enhance the cellular mechanisms and physiological process. Above the threshold, over production of ROS could induce toxicity rather than activation of transcription mechanisms under pathological conditions. Cumulative oxidative damage is involved in the etiology of the above chronic diseases. In order to redox balance, diet-derived bioactive potential could be the novel therapeutic strategy for radical-induce oxidative stress in a given pathological conditions.

1.1. Aims and objectives

The aim of this study was to investigate the possible link between digestion and/or fermentation of bioactive compounds and metabolic responses with focus on the regulation of brain and gut health.

Particularly, this study aimed to investigate the effect of bioactive compound-rich wholemeal cereals (wheat, barley and oats), fruits (blackcurrant) and microalgae *Haematococcus pluvialis* on the -

- Blood glucose response,
- Production of SCFAs (produced by the fermentation of dietary fibre in the gut) and regulation of neural gene expression associated with gut,
- Cellular anti-proliferation, antioxidation, pro-inflammatory cytokine and appetite regulation,
- Expression of risk marker causing neurological disorder.

1.2. Hypotheses

- Incorporation of blackcurrant/microalgae into the wholemeal cereals cookie will enhance textural properties of cookies.
- Improved glycaemia and immunity will be achieved by blackcurrant/microalgae inclusion through enhancing the bioactive compounds, fibre and polyphenols.
- Bioactive compounds will beneficially modulate gut health through enhancing the growth of beneficial bacteria and their metabolic end products will improve satiety.
- Cookie digesta and/or extracts will be effective in cellular anti-proliferation, antioxidation, pro-inflammatory cytokine production and satiety regulation.
- Metabolites of digested and fermented cookie will improve neuronal protection.



Figure 1.1: Project outline

Chapter 2

Literature review

2.1. Diet induced obesity and chronic diseases

Comprehensive epidemiological data and animal studies have consistently shown that the overweight and/or obesity is a major underlying risk factor for the development of metabolic syndrome, insulin resistance, hormone imbalance, dyslipidaemia, neurodegeneration and impairment and/or cessation of bodily or organ functions (Spielman et al. 2014; Desai et al. 2015). Effects of dietary composition on both nutritional (such as carbohydrate, fat, vitamins and minerals) and non-nutritional dietary components (bioactive compounds such as phenolics and fibre) are particularly valuable for body weight regulation (Torres-Fuentes et al. 2015). High glycaemic index (GI) foods composed of mainly readily digestible carbohydrate have been linked to increases in obesity, T2DM, CVD and metabolic syndrome (Zeevi et al. 2015). The glycaemic response of carbohydrate foods depends upon both the type of the food, and the extent of food processing. Numerous health benefits including improved glycaemic control, decreased insulin demand and blood lipid levels of low-GI foods are beneficial in the prevention or management of obesity, T2DM and CVD.

Antioxidant deficiencies, and the response mechanisms of elevated level of ROS mediated oxidations, are the prime causes of major chronic diseases. Redox biology, low concentrations of tissue oxidants (free radicals known as reactive oxygen species, ROS including peroxide; superoxide, $O_2^{\bullet-}$; hydroxyl oxygen, OH^{\bullet} , H_2O_2 , NO and singlet oxygen) are necessary for intracellular signalling. However, overproduction of these highly reactive ROS that bind with nearby molecules (chain reaction) deteriorate the cellular integrity, chemical and/or structural functions including damage DNA and protein (Chio & Tuveson 2017). In order to redox balance, biologically active compounds particularly polyphenol might have a role in the alleviating oxidative damage. The antioxidants may defence against oxidative stress and support cellular antioxidant defences particularly by scavenging radical

(Bagchi et al. 2014). Several studies have reported that secondary metabolites particularly antioxidant polyphenols can cross the blood brain barrier and exerts neuroprotective effects *via* modulation of NF- κ B pathway. Recent research has demonstrated that the synergy of complex mixture of phenolic compounds in foods improves the cellular response to oxidation and exerts protective health effects (Figueira et al. 2017). Hence, phenolics from a variety of sources, for example whole grains, fruits and vegetables are recommended.

Dietary fibre (DF) and polyphenols in foods are two of the most important factors that affecting the rate of glucose release to a carbohydrate food by interaction with the digestive enzymes and slowing gastric emptying (Quirós-Sauceda et al. 2014). Food processing such as grinding and cooking may also affect the carbohydrate digestibility and glucose release (Nayak et al. 2014). A high-fibre diet has received substantial attention in health outcomes. Numerous studies have reported that significant amounts of fibre in a diet can reduce the weight gain (Tucker & Thomas 2009; Grooms et al. 2013), however some reports dispute this (Iqbal et al. 2006). A large number of studies have shown that high-fibre food exerts the potential to lower blood cholesterol and improve insulin sensitivity (Juntunen et al. 2003). DF may produce a distension of the gastrointestinal tract and interacts with the gut microbiota and impacts metabolism and host physiology (Tremaroli & Bäckhed 2012). DFs are also increased bulking and viscosity agents and protect against colon cancer by reducing the carcinogens contact with epithelial cells (Zeng et al. 2014). Numerous interpersonal differences such as lifestyle, genetics, insulin sensitivity and gut microbiota may affect in blood glucose levels (Stanhope et al. 2009; Saxena et al. 2010; Dunstan et al. 2012).

DF escapes upper gastrointestinal digestion, which are fermented by the gut microbes. DF is known to influence the microbial community in gut and alter their metabolites production particularly short-chain fatty acids such as acetate, propionate and butyrate, which are the main energy source of colonocytes (Tremaroli & Bäckhed 2012). Pathophysiologic evidence suggested that the effects of microbial metabolites SCFAs extend beyond the gut. Recent studies have demonstrated that a link between microbial metabolites SCFAs in microglia alteration, neural development, cognition and behaviour (Frost et al. 2014; Erny et al. 2015). DF reduces absorption efficiency and improves satiety

by prolonging gastric release and emptying time, which may alter production of ghrelin and cholecystokinin (Serrano et al. 2012). However, numerous animal studies have demonstrated that different food induces multiple signals in the gut, and that this expression is important for digestion and altered production of gut hormones responsible for satiety and appetite regulation (Chambers et al. 2015; Morrison & Preston 2016). Thus, potential dietary components that may have impact on the regulation of satiety and thereby possess application in the reduce food intake and body weight. Previously it has been reported that fruit fibre may reduce the risk of breast cancer (Suzuki et al. 2008). However, only a few studies have been investigated the synergistic effects of dietary metabolites on satiety and satiety-related peptides regulation in cell and/or animal model.

2.2. Metabolic syndrome

The metabolic syndrome (MetS) is characterised by a causality of clinical risk factors comprising atherogenic dyslipidaemia (high triglycerides levels and low high density lipoprotein), high blood pressure, impaired fasting glucose, hyperglycaemia, insulin resistance, abdominal obesity, endothelial dysfunction and chronic inflammation (Bonomini et al. 2015; Desai et al. 2015). The cluster of these risk factors in the MetS is a major contributor for the development of cardiovascular diseases (CVD; 2 to 4 times higher the risk people with MetS), type 2 diabetes mellitus (T2DM, 5 times), and certain types of cancer, neurodegeneration and obesity (Vykoukal & Davies 2011; Bonomini et al. 2015; Desai et al. 2015). Dietary factors, particularly nutrient composition of diet (such as quality of dietary fat and type of carbohydrate) has a profound influence on the prevention and development of MetS (Perona 2017). A high-fat (saturated fat) diet increases a production of adipocytokines such as TNF- α , adiponectin and leptin, which has been implicated in the pathogenesis of MetS such as impairs insulin action and blood cholesterol level, and induces inflammation in the liver which has been linked to the progression of T2DM, CVD and certain type of cancer, and metabolic diseases (Saltiel & Kahn 2001; Vykoukal & Davies 2011; Furukawa et al. 2017). Observational studies have suggested that a low glycaemic index (GI) diet is associated with reduction the risk factors of MetS such as reduced triglycerides and LDL cholesterol level and improve C-reactive protein (c marker for systemic inflammation) insulin sensitivity (Wong & Jenkins

2007). The glycaemic effect of a diet may be measured by the determination of the glycaemic load, which is calculated by multiplying the GI by the grams of carbohydrate (Saltiel & Kahn 2001). Different carbohydrate foods exert different blood glucose levels and these have been ranked by GI (Jenkins et al. 1981). Studies have assessed that high-fibre carbohydrate of whole-grain foods exerted low GI and associated with the improvement in insulin sensitivity, minimise postprandial insulin release, and induce satiety (Brennan 2005; Poutanen et al. 2017). An increased intake of fruits rich in bioactive compounds such as polyphenols and flavonoids has been credited to reduce the components of MetS and thereby reduce the risk of associated chronic diseases (Amiot et al. 2016).

2.3. Cardiovascular disease

Worldwide, cardiovascular disease (CVD) is the leading cause of death where obesity is the major risk factor to the prevalence of CVD, and increased mortality and morbidity (Nathan et al. 2009; Nichols et al. 2014; Mozaffarian 2016). Besides an abnormal lipid profile, clustering of multiple risk factors are involved to CVD in obesity including metabolic syndrome such as insulin resistance, hyperinsulinemia, hypertension and hypercholesterolemia to accelerated atherosclerosis (Van Gaal et al. 2006). Pathological studies have shown that the level of atherosclerosis (progressive accumulation of lipid substrate and inflammation of the vessel wall) is associated with the number of, and the severity of CVD. Atherogenic dyslipidaemia consists of elevated very low-density lipoprotein triglycerides and apolipoprotein concentrations, increased LDL (low density lipoprotein) and low HDL (high density lipoprotein) cholesterol level (Grundy 2002). Increased levels of lipid increased oxidative stress *via* activation and transcription factor NF- κ B and/or NADPH oxidase (Furukawa et al. 2017). In metabolic syndrome, obese individuals have shown elevated markers of oxidative stress including reactive oxygen species (ROS), which is associated with chronic inflammation, endothelial cell proliferation and increased vasoconstriction, contributing factors to CVD (Huang et al. 2015). Increasing evidence has shown that the dietary composition is highly variable and complex trait, which has been shown to manipulate the risk of CVD. For example diet lower in bioactive compounds (such as fibre and phenolics), and high in fat and sugar-induced metabolic syndrome (high-glycaemic load) exacerbates vascular damage, atherosclerosis and

increased the risk of CVD (Lozano et al. 2016). Cellulose, Hemicellulose, pectin and algal polysaccharides lower plasma triglycerides and LDL cholesterol (Gunness & Gidley 2010). Fermented metabolites of DF, particularly SCFA propionate reduce plasma cholesterol by inhibiting hepatic cholesterol metabolism (Chen et al. 1984). Dietary fibre from wholemeal cereals helps to reduce the risk of CVD by reducing the LDL and serum total cholesterol (Brennan & Cleary 2005; Maćkowiak et al. 2016). Dietary components in fruits and vegetables such as phenols, flavonoids, carotenoids, vitamins and fibre beyond basic nutrition have also been associated to reduce risk for CVD through a number of mechanisms including, reducing oxidative stress, controlling blood pressure, lowering blood cholesterol level and stimulating the immune system (Brennan & Cleary 2005; Alissa & Ferns 2017). Antioxidant polyphenols have been shown to inhibit the formation ROS, which may reduce the risk of atherosclerotic disorder (Husain et al. 2015).

2.4. Diet and cancer

Cancer is the leading cause of death globally, and diet is considered pivotal in the aetiology of cancer, and prevents most cancers (20-60% cancer are related with diet) (Hullar et al. 2014; Stewart & Wild 2017). As the underlying molecular mechanisms of carcinogenesis have been revealed, the link of specific dietary components has attracted much research interest. Therefore, naturally occurring bioactive molecules particularly polyphenols are the current subject of cancer research, due to their preventive potential in neurodegeneration, ageing, liquid malignancies and all form of cancers (Bishayee & Sethi 2016). However, oxidative stress (altered redox status) is the aetiology of cancer disease. Redox biology, low concentrations of tissue oxidants (free radicals known as reactive oxygen species, ROS including peroxide, H_2O_2 ; superoxide, $O_2^{\bullet-}$; hydroxyl oxygen, OH^{\bullet} , NO and singlet oxygen are necessary for intracellular signalling, but overproduction of these highly reactive ROS that bind with nearby molecules (chain reaction) deteriorate the cellular integrity, chemical and/or structural functions including damaging DNA and protein (Panieri & Santoro 2016; Furukawa et al. 2017). Antioxidant deficiencies and the response mechanisms of elevated level of ROS mediated oxidations (oxidative stress) are the prime causes of major chronic diseases. In order to obtain a redox balance, biologically active compounds particularly polyphenol might have a role in the

alleviating oxidative damage. The antioxidants may defend against oxidative stress and support cellular antioxidant defences particularly by scavenging radical (Reinisalo et al. 2015). Moreover, recent research has demonstrated that the synergy of complex mixture of phenolic compounds in foods exerts better protective health effects. Hence, phenolics from a variety of sources for example whole grains and vegetables are recommended.

2.5. Type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM), also known as non-insulin dependent diabetes, is the most commonly diagnosed (about 90 %) of people with diabetes mellitus worldwide (DeFronzo et al. 2015). Diabetes is the most common endocrine disorder (metabolic disease characterised by hyperglycaemia) and the major comorbidity of obesity (Grarup et al. 2014). The underlying abnormality of hyperglycaemia (hallmark of diabetes) is due to the insulin resistance with relative insulin deficiency, defects in insulin synthesis and secretion from pancreatic β -cells and its action on target tissues resulting impaired glucose homeostasis found in most people who develop T2DM (Ueki et al. 2006; Cătoi et al. 2015). Research attention has focussed on the types of dietary carbohydrate intake because of the proposed link between the high glycaemic index/load diet and increased risk of obesity and T2DM. Both the quality and quantity of a diet influence the insulin action (control blood sugar level) and insulin resistance (abnormally high blood sugar level) to the development and progression of T2DM (Hippisley-Cox & Pringle 2004; Brennan 2005). A diet in high carbohydrate intake increases the glycaemic load of foods that stimulates the glucose-sensitive high insulin release in order to maintain glucose homeostasis (Steyn et al. 2004). Longer term glucose-stimulated insulin secretion by a high glycaemic index (high in easily digestible carbohydrate) diet promotes hyperinsulinaemia and decline insulin sensitivity (Steyn et al. 2004; Wilcox 2005). Increased dietary fibre content contributes to a lower glycaemic load of a diet, which leads to the development of fasting blood glucose level and improvement of glycaemic response (Brennan 2005). The complex mixtures of micronutrients, fibre and phytochemicals from wholemeal cereals, fruits and vegetables have been associated to increasing the sensitivity of insulin and regulation of satiety genes expression and reduce incidence of T2DM (Garnett et al. 2014).

2.6. Metabolic control: Gut to Brain

As interest in the brain-gut microbiota interaction has emerged as a prominent factor in the regulation of cognitive functions and the pathogenesis of brain-gut disorders, attention has turned to the impact of microbial composition and their metabolic functions on the brain. More than 100 trillion of resident bacteria within the human gut are extremely diverse, and they influence the host's physiological, metabolic, immunological and neurological functions (Borre et al. 2014; Maukonen & Saarela 2015). Colonization of the gut has two major health benefits to the host; firstly, regulator of the immune system and secondly, act as a metabolic organ to maintain the whole-body energy homeostasis (Peterson & Artis 2014). Alterations to the balance of microbial composition in the gut may alter the bidirectional brain-gut interactions that promote the host susceptibility to the disorder, including gastrointestinal disorder, irritable bowel syndrome (IBS); metabolic disorder such as impaired glucose control, insulin insensitivity and neurological disorder such as cognitive decline, Parkinson's disease, Alzheimer's disease (Petra et al. 2015; Boulangé et al. 2016; Sampson et al. 2016). The gut bacterial taxa are modulated by diet (David et al. 2014). Long-term dietary changes, such as consumption of high-readily digestible carbohydrates, high-indigestible carbohydrate, high-fat, high-fibre and polyphenols are the key modulators that can affect the microbial profile and changes their metabolic end-products (Maukonen & Saarela 2015). Research has shown that the dietary fibre can regulate the metabolism of gut microbiota and have a beneficial effect to stimulate the growth of beneficial bacterial, as they are fermented by the intestinal microbes (Koh et al. 2016). Thus, dietary interventions are a potential modulator to regulate gut microbial composition and host health (Dewulf et al. 2012). Epidemiological studies have shown an inverse relation between the high fibre intake and a reduced risk of IBS, CVD, T2DM and colon cancer (Reicks et al. 2014; Consortium 2015; Rao et al. 2015b). Short-chain fatty acids (SCFAs) acetate, propionate and butyrate are the major bacteria-derived metabolic end-products of dietary fibre fermentation. The higher microbial SCFAs production, lower the pH value in the colon changes the bacterial composition and inhibit the growth of pH sensitive pathogenic bacteria (Duncan et al. 2009). SCFAs are acting as a signalling molecule to regulate fatty acid synthesis, oxidation and lipolysis, which promotes the lipid and

glucose metabolism, maintains the plasma fatty acids concentration, inhibits fat storage, increases satiety and decreases body weight (Koh et al. 2016; Rooks & Garrett 2016).

2.7. Health factors of whole grains

Whole grains are important source of nutrients, but they are also rich source of biologically active compounds predominantly dietary fibre (almost double compared to vegetables on a dry matter basis), vitamins, minerals and phytochemicals including antioxidants phenolic compounds which is in accounts for 15 % of total grain weight (Rebello et al. 2014; Beloshapka et al. 2016). Mechanistically, whole grains might have ability to trigger a biochemical cascade that modulate glycaemia and extend satiety. The underlying mechanism might be delayed rate of nutrient release and extended signals to the gut hormone. Whole grain fibres (soluble/ insoluble) are associated with the development of healthy gut microbiota, enhanced production of short-chain fatty acids (such as acetate, propionate and butyrate), and involved in physiological mechanisms such as improved satiety, insulin sensitivity, decreased gastric emptying rate and help to control body weight (Bach Knudsen 2015; Roager et al. 2018). Whole grain fibres are also agents which promote bulking and viscosity and protect against colon cancer by reducing the carcinogens contact with epithelial cells (Lindberg 2014). In addition to dietary fibres whole grain contain high amount of phytochemicals, phenolic acids, carotenoids, flavonoids, n-3 fatty acids, phytic acids and vitamin E are well-known biologically active antioxidants, which is substantially reduced in refined cereals due to removal of cell walls (Lu et al. 2014). Mounting evidence have hypothesized that the most food bioactive compounds have potential anticancer properties which is mainly attributed to the phenolic compounds, but they may be present in in free and/or bound forms found in the cell walls (Vitaglione et al. 2014; Pang et al. 2018). For example, ferulic acids may be bound to the hemicellulose fibre (Turner et al. 2015).

2.8. Health factors of blackcurrant berry (*Ribes nigrum*)

Bioactive molecules such as polysaccharides and polyphenols are widely distributed in plant foods for example wholegrain cereals and fruits. High consumption of bioactive compounds rich fruits and vegetables may be associated with protective health effects and disease prevention. Blackcurrant is one of the most biologically rich berries among all berry fruits, which exerts protective potential

against memory dysfunction and oxidative stress damage (Benn et al. 2015; Esposito et al. 2015). It has been widely accepted that dietary polyphenols exhibit inhibitory activity on enzymes *in-vitro*, obstruct digestion and absorption (Jakobek 2015). High contents of phenolics and flavonoids present in blackcurrant potentially increase lipolysis and reduce triglycerides levels, which is associated with reduction of heart diseases (Benn et al. 2015). Mounting evidence has suggested that bioactive berry phenolics are inversely associated with the risk of cancer, type 2 diabetes and obesity-related disorders (Anhê et al. 2015; Amiot et al. 2016). Even though the antioxidant effects of blackcurrant berry have been widely studied, there is a lack of data available about the biological activities linking blackcurrant to the regulation of satiety signals in order to facilitating weight loss.

2.9. Health factors of microalgae *Haematococcus pluvialis*

Microalgae such as *Haematococcus pluvialis* are regarded as untapped natural resources that have high aggregated bioactive compounds such as carotenoids, vitamins, minerals, sterols, fatty acids, phenolics and flavonoids. *H. pluvialis* microalgae is a rich sources of antioxidant astaxanthin, which exerts several fold higher radical scavenging capacity than β -carotene and vitamin E, have been demonstrated in several *in-vitro* and animal studies (Régnier et al. 2015; Yu et al. 2015; Liu et al. 2018). Microalgae bioactive compounds are structurally and biologically unique, which have been demonstrated anti-inflammatory and antitumor effects (Liu et al. 2018). Some of the recent studies have reported that bioactive molecules from microalgae are potent against HIV, cancer and hepatic diseases (de Morais et al. 2015; Shahidi & Ambigaipalan 2015). Bioactive compounds of *H. pluvialis* microalgae particularly astaxanthin protect against photo-induced oxidation, ulcer's *helicobacterpylorii* infections, LDL-cholesterol and carcinogenesis (Rao et al. 2015a; Bilbao et al. 2016). However, a few toxicological trials on a limited number of marine algae have shown the toxicity from the ingestion of microalgae varies widely. The toxicity of microalgae might be species specific, but needed for an extensive investigation on human cell model.

Chapter 3

Materials and methods

3.1. Chemicals and reagents

Gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2'-azobis (2-amidinopropane) dihydrochloride (ABAP), 2',7'-dichlorofluorescein diacetate (DCFH-DA) and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich Ltd. (St. Louis, MO, USA). Chlorogenic acid, *p*-coumaric acid, ferulic acid, vanillic acid, caffeic acid, 3,4-hydroxybenzoic acid and quercetin were purchased from Aladdin Industrial Corporation (Shanghai, China). Chromatographic grade of acetonitrile and methanol were purchased from ANPEL Scientific Co. Ltd. (Shanghai, China). William's medium E (WME), fetal bovine serum (FBS), insulin and other cell culture reagents were purchased from Gibco Biotechnology Co. (Grand Island, NY, USA).

3.2. Sample collection and preparation

Blackcurrant (*Ribes nigrum*) powder was provided by Sujon Berries (Nelson, New Zealand). Dried (convection drying oven at 55 °C for 72 hours) microalgae *H. pluvialis* was provided by Supreme Biotechnologies Ltd. (Nelson, NZ). All samples were ground using a grinder (Sunbeam AutoGrinder, M-EM0415, China) and sieved through a 0.5-mm screen to obtain standard particle sizes. Wholemeal wheat (Champion Flour, Auckland, NZ), barley (Ceres Organics, Auckland, NZ) and oat flour (Ceres Organics, Auckland, NZ) were purchased locally.

3.3. Cookie preparation

Cookies were prepared following the American Association of Cereal Chemists (2000) method 10-50D with slight modification. All ingredients (except flour and blackcurrant powder) – sugar (Chelsea white sugar, New Zealand Sugar Company Ltd. Auckland, New Zealand), salt (Cerebos iodized table salt, Cerebos Gregg's Ltd, Sydney, Australia) and sodium bicarbonate (Edmonds, Goodman Fielder Ltd, Auckland, New Zealand) were mixed with vegetable shortening (Kremelta, Peerless foods, Victoria, Australia) in an electric mixer (Breville the kinetix wize, BFP450, NSW, AU) on speed 1 for 3

min. Distilled water and dextrose solution (8.9 g dextrose hydrous in 150 ml water) were added and mixed for a further 1 min on speed 2 with scraping down every 30 s. The flours (Wholemeal wheat flour, Champion Flour, Auckland, NZ; Oat flour, Ceres Organics, Auckland, NZ; Barley flour, Ceres Organics, Auckland, NZ) and blackcurrant powder or astaxanthin powder were added and mixed for 2 min with scraping down every 30 s. The control samples were prepared using wholemeal flour and in experimental samples blackcurrant powder or astaxanthin powder replaced (5 %, 10 % and 15 %) (Table 3.1) wholemeal wheat, barley and oat flours. The cookie dough was rolled to a thickness of 6 mm using aluminium guides and cut with a cookie cutter of 57 mm diameter. The cookies were placed in metal trays and baked at 180 °C in an electric oven (BAKBAR turbofan convection oven, E311, Moffat Pty Ltd, AU) for 8 minutes. Cookies were cooled at room temperature before being wrapped in foil, placed in air tight polyethylene bags, and stored at ambient conditions.

Table 3.1: Formulation of cookie

Sample	Wholemeal flour	Blackcurrant/astaxanthin powder	Other ingredients
Control	225.00	-	Vegetable shortening (64.0 g),
5% Blackcurrant or astaxanthin	213.75	11.25	Sugar (130 g), Salt (2.1), Sodium bicarbonate (2.5 g), Dextrose solution (33 g) , Water (16 g)
10% Blackcurrant or astaxanthin	202.5	22.5	
15% Blackcurrant or astaxanthin	191.25	33.75	

3.4. Physical characteristics

The cookie diameter (mm) and thickness (mm) were measured using callipers (INSIZE digital caliper, series 1112, INSIZE INC, USA). The colour of the cookie samples were measured in terms of CIE L^* , a^* and b^* systems by using a colorimeter (Konica Minolta, Chroma Meters CR-210, Japan). The colour differences of the cookies were calculated by the following equation

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

3.5. Moisture

Moisture content of the cookie samples were calculated after drying ground cookie samples (2 g) overnight in an oven at 105 °C (AOAC 1990).

3.6. Texture

The hardness of the cookies (fracture force) was measured by using a texture analyser (TA.XT plus Texture Analyser, Stable Micro Systems, UK) with a 3-point bend rig probe. The analyser was set at a load cell 50 Kg; pretest speed 2 mm/s; test speed 5 mm/s; posttest speed 10 mm/s; return to start mode. The whole cookies were placed on the support ring and the probe moved downward until the samples were broken. The peak force (Kg) was recorded.

3.7. Simulated digestion of the cookie samples

In-vitro carbohydrate digestion and multiphase simulated enzymatic digestion were carried out to determine the glycaemic response and the cellular biological activity of the cookie digesta.

3.7.1. *In-vitro* carbohydrate digestibility and glycaemic response

The *in-vitro* digestion method adopted by Foschia et al. (2015) was used to evaluate carbohydrate digestibility of the cookie samples. This method assesses the amount of free glucose during the test time in incubation condition by the enzymatic hydrolysis. The samples (0.5 g) were dissolved in 30 ml of RO water and held at 37 °C for 10 min with constant stirring. Then, 1 mL pepsin solution (1 g pepsin in 10 mL 0.05 M HCl) was added as a gastric digestion for 30 min. Aliquots (1 mL) were taken (Time 0) and added to 4 mL absolute alcohol to stop further reaction. Amyloglucosidase (0.1 mL) was added in order to prevent end product inhibition of pancreatic α -amylase. Pancreatin (5 mL of 2.5% solution in 0.1 M sodium maleate buffer) was added to represent ileal digestion and 1 mL aliquots were taken after 20, 60 and 120 min, and added to 4 mL absolute alcohol. The samples were stored at 4 °C until the subsequent reducing sugar analysis. Sugars released were measured using the 3,5-dinitrosalicylic acid (DNS) colorimetric method. The amount of glucose released was calculated in mg of glucose/g of sample using the absorbance values and area under the curve (AUC) was calculated by dividing the graph into trapezoids.

3.7.2. Multiphase simulated enzymatic digestion

An *in-vitro* simulated digestion method developed previously (Papillo et al. 2014), was followed with some modification for four sequential phases enzymatic digestion. The total digestion process was carried out at 37 °C in a water bath. The samples (2.0 g) were completely dispersed in 20 mL RO water. Then, 0.5 mL of α -amylase solution (6.5 mg enzyme in 5 mL of 1 mM CaCl_2 ; pH 7.0) was added to each sample and incubated for 10 min. The pH was then adjusted to 3 with 6 M HCL for the gastric phase digestion. Pepsin (0.1 g) was added to the oral digesta and incubated for 2 h. Then, the pH of the digesta was adjusted to 7.5 with 0.9 M NaHCO_3 for the intestinal process. The enzyme solution (5 mL of 0.1 g pancreatin, 0.6 g lipase and 0.625 g bile extract in 0.1 M NaHCO_3 ; pH 7.5) was added to represent small intestinal digestion and incubated for 4 h. For the large intestinal digestion, the pH was adjusted to 4.0 and 60 μl viscozyme L was added to the digesta, then, incubated for 6 h to simulate colonic digestion. Aliquots (2 mL) of each physiological phase digested were collected and stored at – 40 °C until further analysis.

3.8. Determination of total phenolic content

Total phenolic compounds (TPC) of the samples were extracted with 70 % methanol and measured by Folin-Ciocalteu reagent as described method by Floegel et al. (2011). Samples (1 g, dry basis) were extracted with 70 % methanol (20 mL, in a plastic pot placed in the multi-stirrer at 2 speed overnight) and then centrifuged at 700 RCF for 10 min. The supernatant was used to determine the total phenolic content. Freshly prepared 2.5 mL of 0.2 N Folin-Ciocalteu's reagent and 7.5% Na_2CO_3 was added to the extract sample (0.5 mL) and then incubated for 2 hours in the dark. The absorbance of the reaction mixture was measured at 760 nm. Gallic acid was used as a standard to determine total phenolic of the samples as mg gallic acid equivalent (GAE)/g sample.

3.8.1. Phenolics extraction from antioxidant-dietary fibre

Free and bound phenolics from each sample was extracted separately according to the method developed by Guo et al. (2012).

3.8.1.1. Free Phenolics

TDF fraction (1.0 g of each sample) was blended with 20 mL of 80 % chilled acetone for 5 min, homogenized with a homogenizer for another 3 min and centrifuged at 4577 RCF for 10 min. Finally the supernatants were pooled in a flask. The process was repeated twice and the collected supernatants were evaporated at 45 °C through rotary evaporator to dryness. The extracts were reconstituted with 10 % methanol and stored at -40 °C until further analysis.

3.8.1.2. Bound phenolics

The residues from free phenolics were digested with 20 mL of 4 M NaOH while shaking under nitrogen for 1 h at room temperature. The mixture was acidified to pH 2.0 using concentrated HCL and then extracted with ethyl acetate for five times (each time homogenize for 1 min and then centrifuged at 4577 RCF for 5 min). The ethyl acetate fraction were evaporated at 45 °C to dryness and then reconstituted with 10 % methanol and stored at -40 °C until further analysis.

3.9. Chemical antioxidant analysis

The antioxidant capacity of samples was determined by DPPH and ORAC assays.

3.9.1. DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The scavenging capacity of the samples was determined using DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical as described method by Floegel et al. (2011). In brief, freshly prepared 1 mL of 0.1 mM methanolic DPPH solution was added to 0.5 mL of the sample and incubated for 30 min in the dark. The absorbance of the reaction mixture was measured at 517 nm. Trolox was used as standard and the DPPH radical scavenging capacity was expressed as $\mu\text{mol Trolox equivalent (TE)/g sample}$.

3.9.2. ORAC (Oxygen radical absorbance capacity) assay

ORAC (Oxygen radical absorbance capacity) was determined as described by Floegel et al. (2011) with some modification. Briefly, 25 µL diluted samples and 150 µL of 10 nM fluorescein were pipetted into each working well of the microplate and incubated at 37 °C for 30 min. Next, freshly prepared 25 µL AAPH (2,2'-azobis (2-amidinopropane) dihydrochloride) solution was added to the preincubated microplate. Microplate Reader (FLUOstar Omega [0403], microplate reader, BMG LABTECH, Germany) was used for all measurements. Fluorescence was measured (at wavelengths: excitation 485 nm; and emission 510 nm) from the bottom every 60 seconds for total 60 min. Trolox was used as a standard and antioxidant capacity was expressed as mmol Trolox equivalent (TE)/g sample calculated automatically using (Omega MARS data analysis software, program version 3.02 R2).

3.10. Determination of phenolic acids composition by RP-HPLC

The phenolic acid composition of samples were identified and quantified by chromatographic analysis as described previously (Teleszko et al. 2015). The HPLC (Waters Co., Milford, MA, USA) system was equipped with a Sunfire C18 reverse phase column (250 x 4.6 mm, 5 µm particle size, Waters, USA) at 280 nm wavelength. The samples were eluted with a gradient system consisting of binary elution phase A (0.1 % trifluoroacetic acid in Milli-Q water) and phase B (acetonitrile) with a flow rate 1.0 mL/min. The gradient conditions were 0-5 min 10 % B, 5-20 min 25 % B, 20-25 min 35 % B, 25-40 min 90 % B, 40-50 min 10 % B, 50-60 min 10 % B. The chromatographic peaks were analysed by their retention times and compared with the pure standard compounds in specific wavelength. The contents of phenolic acids were determined and results were expressed as mg/100 g DW.

3.11. Determination of insoluble and soluble dietary fibre (IDF, SDF)

The cookies were analysed for the IDF and SDF following an AOAC (1995) official method (AOAC 991.43) (AOAC 1995). A fibre assay kit (K-TDFR 12/15) (Megazyme International, Bray, Ireland) was used to determine the samples fibre content. Briefly, cookies were ground to 300-400 µm and

completely dispersed in MES-TRIS buffer. Enzymatic digestion (1 g sample: 50 µL heat-stable α-amylase at 98-100 °C, for 30 min. Followed by 100 µL protease at 60 °C for 30 min ; 5 mL 0.561 N HCl was added to adjust pH 4.1-4.8, followed by 200 µL amyloglucosidase and incubated at 60 °C for 30 min with constant agitation). The samples were quantitatively transferred to a glass crucible containing celite mat for filtration. The residue was washed with RO water, 95 % diluted ethanol and 100 % acetone (IDF). Four volumes of pre-heated 95 % ethanol of 60 °C was added to the filtrate and water washings and allowed to stand for one hour at room temperature. The resulting precipitate was filtered to a glass crucible containing celite mat. The residue was washed 95 % diluted ethanol and 100 % acetone (SDF). IDF and SDF residue were dried in 103 °C oven overnight and weight was measured. Then the samples were ashed in a muffle furnace at 525 °C for 5 h and re-weighed. Total nitrogen of the residues (IDF and SDF) were analysed as previously described (Bremner 1965) to determine the protein content using the conversion factor of 6.25.

3.12. Total dietary fibre (TDF) isolation

According to the AOAC (1995) official method (AOAC 991.43) (AOAC 1995), total dietary fibres were isolated from the cookie samples with filtration modification. Buchner funnel and filter paper 541 (Whatman International Ltd. Maidstone, UK) were used instead of a glass sinter crucible and celite mat when isolating the fibre (Titgemeyer et al. 1991), as a substrate for the fermentation. TDF was isolated from the wholemeal wheat cookie (WCC), wholemeal wheat + 15 % blackcurrant (W15B), wholemeal wheat + 15 % astaxanthin (W15A), wholemeal barley cookie (BCC), wholemeal barley + 15 % blackcurrant (B15B), wholemeal barley + 15 % astaxanthin (B15A), wholemeal oat cookie (OCC), wholemeal oat + 15 % blackcurrant (O15B) and wholemeal oat + 15 % astaxanthin (O15A).

3.13. Bacterial strains

Lactobacillus rhamnosus (ATCC 7469) (LR), *Lactobacillus acidophilus* (ATCC11975) (LA), *Bifidobacterium breve* (ATCC15700) (BB), *Bifidobacterium longum* (ATCC15707) (BL) were collected from Institute of Environmental Science and Research (ESR), Christchurch, New Zealand. These bacterial strains were selected for the fermentation as they are beneficial microbes in human gut and have been identified as probiotics (Martinez et al. 2015).

3.14. Bacterial sub-culture and preparation of cell suspensions

Freeze dried *Lactobacillus* spp. were rehydrated by subculturing in MRS medium as previously recorded (de Man, Rogosa and Sharpe) (Oxoid Ltd., Hampshire, England), and *Bifidobacterium* spp. reinforced clostridial medium under strict anaerobic conditions. *Lactobacillus* spp. for 24 h and *Bifidobacteria* spp. for 72 h were incubated at 37 °C to obtain the complete growth curves of the microbes. Pure cultures of all bacterial strains (10^7 cfu/mL) were mixed in a 1:1:1:1 (v/v) ratio to prepare equal proportions. The bacterial cells were combined and centrifuged at low speed then washed with saline (0.85 % NaCl) to remove excess carbon, and resuspended in the PYF (Peptone Yeast extract Fildes) carbohydrate-free basal medium.

3.15. Preparation of PYF growth medium

Carbohydrate-free PYF basal medium was used for the *in-vitro* fermentation (Yoshimoto et al. 2005). The medium consisted of 10 g Trypticase Peptone, 5 g yeast extract, 0.5 g L-cysteine hydrochloride, 40 mL digested horse blood and 40 mL salt solution (1 L salt solution (pH 7.6) contained: 0.2 g CaCl₂, 0.2 g MgSO₄.7H₂O, 1.0 g KH₂PO₄, 1.0 g K₂HPO₄, 10 g NaHCO₃, and 2.0 g NaCl).

3.16. *In-vitro* fermentation

Fermentation was carried out in 100 mL sterile bottles. Each bottle contained culture medium, substrate and mixed culture. Culture medium (50 mL), 1 % (v/w) substrate (mL/mg) (extracted TDF from cookie) was added to each bottle and sealed for 24 h at room temperature for complete hydration of the fibre. The bottles were incubated at 37 °C for 2 h prior to inoculation. A precultured combination of bacterial suspension (5 mL, 10^7 cfu/mL) was added to each bottle and incubated at 37 °C in strict anaerobic conditions (in the anaerobic jar with gas pack). A negative control (medium only) and a positive control (glucose) were also fermented. Fermentation was carried out in duplicate. Aliquots (2 mL) were taken for SCFAs analysis at 0, 6, 24 and 48 h incubation respectively. The microbial growth was evaluated by the optical density (OD₆₀₀, after centrifugation at 700 RCF for 5 min) and measuring the pH as it was lowered. The decrease in pH is caused by acid production of the growing microbes.

3.17. Determination of SCFA

SCFAs (acetate, propionate and butyrate) analysis was conducted as described previously (Al-Marashdeh et al. 2016). Aliquot samples were thawed and centrifuged at 10000 RCF for 30 min at 4 °C. The supernatant (100 µl) was transferred to an eppendorf tube and vortex-mixed with 20 µl of standard and 40 µl meta-phosphoric acid (31 %). After 30 min incubation at room temperature, samples were centrifuged at 10000 RCF for 15 min at 4 °C. The supernatant were transferred into an autosampler (AOC-20i) vial and injected onto a SGE BP21 30 x 530 µm x 1.0 µm wide-bore capillary column to a GC (Shimadzu GC-2010).

3.18. Human cell culture and culture condition

HepG2 (human hepatocellular liver carcinoma) cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and routinely propagated as stated previously (Zhu et al. 2015). Cells were grown in conditioned growth medium (CM) consisted of Williams medium E (WME) supplemented with 5 % fetal bovine serum (FBS), 10 mM N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 2 mM L-glutamine, 5 µg/mL insulin, 0.05 µg/mL hydrocortisone, 50 units/mL penicillin, 50 µg/mL streptomycin and 100 µg/mL gentamycin. The culture was maintained at 37 °C and 5 % CO₂ in an incubator humidified condition and cells were passaged after 75 % confluence.

E22G-mCherry Hek 293 cell lines was provided by University of Cambridge, England. The cells were sub-cultured in DMEM medium supplemented with 10 % FBS, 5 mol/L L-glutamine, 50 µg/mL blastidin in humidified condition (37 °C and 5 % CO₂).

3.19. Cellular antioxidant activity (CAA)

The CAA assay was conducted based on the method previously developed by Wolfe and Liu (2007). Briefly, HepG2 cells (passages between 12 and 35) were seeded at a density 6 x 10⁴ cells/well on black 96-well microplates (#3603, Corning, NY, USA). After 24 h incubation (37 °C in a humidified atmosphere of 5 % CO₂), cells were washed with PBS and then triplicate wells were treated with 50

μL of quercetin (antioxidant standard) and digesta/antioxidant-DF extracts with 50 μL of 25 μM DCFH-DA dissolved in antioxidant treatment medium (WME supplemented with 2 mM L-Glutamine and 10 mM HEPES). 50 μL of antioxidant treatment medium and 50 μL of DCFH-DA solution (25 μM final concentration) were added to control and blank (negative control) wells. After 1 h incubation, treatment medium was removed from well and cells were washed with PBS (PBS wash protocol) or without wash (no PBS wash protocol), 100 μL of freshly prepared 600 μM of ABAP dissolved in oxidant treatment medium (Hank's Balanced Salt Solution without phenol red + 10 mM HEPES) were added to each well. 100 μL of oxidant treatment medium only were added to blank well. The plate was read immediately in multi-mode microplate reader (FilterMax F5, Molecular Devices, USA) with fluorescence intensity at excitation of 485 nm and emission of 535 nm every 5 min for 13 times. The oxidative degeneration of DCFH-DA to DCF, CAA values were calculated using the following equation,

$$\text{CAA unit} = 1 - (\int\text{SA} / \int\text{CA})$$

Where, $\int\text{SA}$ and $\int\text{CA}$ are the integrated area under the fluorescence versus time curve of sample and control respectively.

The median effective dose (EC_{50}) of each sample was calculated from the median effect plot of $\log(fa/fu)$ versus $\log(\text{dose})$, where, fa and fu are the fraction affected (CAA unit) and unaffected (1-CAA unit) by the treatment respectively. The EC_{50} values were converted to CAA values and data was expressed as micromoles quercetin equivalent per 100 g of sample ($\mu\text{mol QE}/100 \text{ g sample}$).

3.20. Determination of cytotoxicity and anti-proliferative activity

The cytotoxicity of digested samples were investigated by modified methylene blue assay (Felice et al. 2009). Briefly, precultured HepG2 cells were seeded in 96-well microplate at a density 4×10^4 cells/well and incubated in humidified atmosphere (37 °C and 5 % CO_2). After 24 h incubation growth medium was removed from the well and washed with 100 μL PBS. Then, 100 μL growth medium containing different concentration of digesta/antioxidant-DF and medium only were applied to the treatment and control cell well respectively, and incubated for another 24 h. After the incubation period, the medium was removed from the wells and they were washed with 100 μL PBS. Cells were stained with 50 μL methylene blue solution (98 % Hanks Balanced Salt Solution (HBSS), 0.67 %

glutaraldehyde and 0.6 % methylene blue) and incubated for 1 h at 37 °C. Cells were then washed with deionized water and briefly dried. 100 µL of elution buffer (49 % PBS, 50 % ethanol and 1 % acetic acid) was added to each well and the plate was placed on a table oscillator for 20 min. The absorbance was measured at 570 nm on a multi-mode microplate reader (Filter Max F5, Molecular Devices, USA). The different concentrations of digested samples were compared to the control, and reduction of cell viability by >10 % was considered to be cytotoxic.

The anti-proliferative efficacy of digested samples was evaluated on HepG2 cancer cell lines following the modified methylene blue assay (Felice et al. 2009). Briefly, precultured HepG2 cells were seeded to 96-well microplate at a density 2.5×10^4 cells/well and incubated for 4 h. After the incubation period the growth medium was removed and the cells were washed with 100 µL PBS. Medium (100 µL) containing different concentrations of digesta and medium only were applied on treatment and control cell well, incubated for 72 h in humidified atmosphere (37 °C and 5 % CO₂). The following steps were the same as described in the cytotoxicity assay. The anti-proliferative effects were assessed by the EC₅₀.

3.21. Quantitative real-time PCR and regulation of gene expression

In order to investigate the bioactivity of fermented metabolites in the regulation of multiple satiety and inflammation related gene expression in HepG2 cells RT-PCR was performed. Seeded cells (1×10^5 cells/mL) were treated with the metabolites (0.5 mM) and incubated for 12 h in culture condition. Total cellular RNA was isolated with RNA Extraction Kit (Dongsheng Biotech, Guangzhou, China) and integrity was determined by spectrophotometer at absorbance $A_{260/280}$ nm. cDNA was generated using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara Biotechnology, Dalian, China), following manufacturer's instructions. The qPCR on resultant cDNA was performed using SYBR^R Premix Ex Taq™ Kit (Takara Biotechnology, Dalian, China) in the Bio-Rad MiniOption™ Real Time PCR System (Bio-Rad, Hercules, CA, USA), using GAPDH as internal control. The target genes primers were obtained from Sigma-Aldrich and the sequences were: glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5'-GTCAGTGGTGGACCTGACCT-3' (forward) and 5'-

AGGGGTCTACATGGCAACTG-3' (reverse); cholecystinin (CCK): 5'-GCCGAGGAGTATGAGTACCC-3' (forward) and 5'-TTCTGGTGAGGTGTGTGGTT-3' (reverse); glucagon-like peptide-1(GLP-1): 5'-AGGTCTTGCCACTCTCATC-3' (forward) and 5'-CAGCGGGTGTGATGATTCTG-3' (reverse); gastrin-releasing peptide (GRP): 5'-GGTCACCAGTCTCTGCTCTT-3' (forward) and 5'-GAAGACTCCCCTGTGCTCTT-3' (reverse); nucleobindin-2 (NUCB2)/nesfatin-1: 5'-AGTGGGAGGCTAAGCAAAGA-3' (forward) and 5'-CAGATCACTTGTTGCCGCTT-3' (reverse); peroxisome proliferator-activated receptor γ (PPAR γ): 5'-AAATGCGAGAGTGGGAAGGA-3' (forward) and 5'-AGGGTCTTGCTATGTTGCCT-3' (reverse); interleukin-6 (IL-6): 5'-GTGTGAAAGCAGCAAAGAGGC-3' (forward) and 5'-CTGGAGGTACTCTAGGTATAC-3' (reverse); interleukin-1 β (IL-1 β): 5'-AAAAGCTTGGTGATGTCTGG-3' (forward) and 5'-TTTCAACACGCAGGACAGG-3' (reverse); nuclear factor-kappa B (NF-kB): 5'-CAAAGTAGACCTGCCAGAC-3' (forward) and 5'-GACCTCTCTAATCAGCCC-3' (reverse). Relative mRNA expression level was calculated using $2^{-\Delta\Delta Ct}$ method, (where, $\Delta Ct = Ct$ (gene of interest) – Ct (housekeeping gene); $\Delta\Delta Ct = \Delta Ct$ (sample) - ΔCt (control average); fold of gene expression = $2^{-\Delta\Delta Ct}$).

3.22. Determination of A β -aggregation (image analysis)

E22G-mCherry Hek 293 cells were plated onto a 24-well plate at equal density (4×10^3 /mL) in the culture medium. After 24 h incubation, the cells were treated with the tested samples of two different SCFAs concentrations (0.1 and 0.5 mM) for 48 h in humidified culture condition. Then the cells were induced until the aggregation appears (72 h-96 h). The fluorescence images of the plated cells were photographed under fluorescence microscope (OLYMPUS, Japan) and the aggregation rate of each sample was calculated by the following formula:

$$\text{Aggregation Rate (\%)} = \frac{\text{Number of aggregates}}{\text{Number of cells}} \times 100\%$$

The final aggregation rate of each group was carried out by comparing to the control group.

3.23. Determination of A β -aggregation (flow cytometry analysis)

The seeded cells (density 3×10^4 /mL) were treated with the tested samples of two different SCFAs concentrations (0.1 and 0.5 mM) for 48 h in humidified culture condition. Then, the cells were

induced until the aggregation appears (72 h-96 h). Finally, the cells were harvested by trypsinization and collected by centrifugation at 1000 ×g. Multiparameter flow cytometry was used to determine the A β -aggregation among cells.

3.24. Statistical analysis

All analysis was carried out in triplicate. Results are presented as mean \pm standard deviation (SD). Statistical differences of multiple treatment groups were determined by analysis of variance (ANOVA) using Tukey's multiple comparison test with statistical software Minitab (version 17, Minitab Inc). Dose-effect analysis was employed using Calcsyn software (Version 2.0, Biosoft, Cambridge, UK). Significant differences were considered at P-values of ≤ 0.05 .

Chapter 4

The combined effect of blackcurrant powder and wholemeal flours to improve health promoting properties of cookies

Hossain, A., M. A. Brennan, S. L. Mason, X. B. Guo and C. S. Brennan (2017). "The Combined Effect of Blackcurrant Powder and Wholemeal Flours to Improve Health Promoting Properties of Cookies." Plant Foods for Human Nutrition **72**(3): 280-287.

Abstract

A diet with high glycaemic index, which causes rapid spikes in blood sugar level, can lead to disorders such as - significantly increased risk for type 2 diabetes, cardiovascular disease and obesity. These conditions are also linked to the progression of cognitive decline and neurodegenerative diseases including Alzheimer's disease. The role of dietary fibre (DF) in disease prevention has been investigated extensively and prospective studies observed that increased DF intake decreased the risk of cardiovascular disease, reduced the risk of weight gain as well as the risk of type 2 diabetes, colon cancer and ameliorated brain and gut health. Blackcurrant powder (BC) is a rich source of dietary fibre and bioactive compounds among all berries. Wholemeal wheat, barley and oat flour contain high amount of fibre. In this study we have developed a model food cookie to investigate the glycaemic glucose equivalent (GGE) *in-vitro* and antioxidant activities of three different wholemeal flours (wheat, barley and oat) with different replacement level (5 %, 10 % and 15 %) of blackcurrant powder. Increasing the proportion of blackcurrant powder in the cookie resulted in a significant ($p < 0.05$) decrease in glucose release after *in-vitro* digestion compared to the control (wheat control 624.4mg/g, W15BC 440.0mg/g; BCC-554.6mg/g, B15BC-435.3mg/g, OCC-523.6mg/g, O15BC-415.9mg/g at time 120 minutes). In addition, incorporation of blackcurrant powder in cookies up to 15 % increased the antioxidant capacity. Several researchers have revealed that the bioactive compounds, especially polyphenols and anthocyanin of fruits have enzymes that have an inhibitory

effect during the digestion process. Therefore, the combination of wholemeal flour and the bioactive compound rich blackcurrant can provide the potential to improve the nutritional value and reduce the glycaemic index of such foods.

4.1. Introduction

Prospective epidemiological studies, randomized prevention/clinical trials and many short-term studies of fundamental pathology such as blood pressure, lipids and glucose level have illustrated that the diet is a leading modifiable determinant of major chronic diseases. Rapidly increasing chronic diseases such as heart diseases, some forms of cancer, and type 2 diabetes have been defined as a global pandemic, as these diseases are responsible for over 60 % of global deaths (Hall et al. 2015). Obesity is a underlying risk factor for those chronic conditions (RP2) but also cognitive decline and other neurodegenerative diseases notably Alzheimer's diseases in later life (Xu et al. 2011). The dietary changes (qualitative and quantitative) "nutrition transition": shift towards a diet, characterized by high in saturated fats, sugar and other refined carbohydrates, and low in dietary fibre and complex carbohydrates (fruits and whole grain products) leads to development of obesity and nutrition related chronic diseases (Drewnowski & Popkin 1997; Brennan & Samyue 2004; Astrup et al. 2008).

In the era of increasing consumer attention to diet and health, it is important to develop new food products - maintaining aspects of traditional nutrition with additional positive health benefits, for example improved blood glucose control and lowering blood cholesterol level. Epidemiological studies and randomized control trials show that dietary modification, in particular whole grains and fruit intake are protective and recovery against cancer, cardiovascular disease, type 2 diabetes and obesity (Aguilera et al. 2016; Allison et al. 2016; Fontes-Villalba et al. 2016; Nelson et al. 2016). Potential mechanisms for these wide ranges of action are due to the rich source of nutrient, dietary fibre, and other bioactive compounds such as polyphenols, flavonoids, anthocyanins and vitamins (Kris-Etherton et al. 2002; Aguilera et al. 2016). Most of the phenolic compounds are potent

antioxidants, which scavenge free radicals and prevent oxidative reaction of pathogenesis (Seeram & Heber 2007).

Whole grains such as whole wheat, barley and oat are not only a concentrated source of carbohydrate but are also rich sources of nutrients such as fibre, protein, vitamins, minerals and phytochemicals. Starch is the predominant carbohydrate in whole grains; it is rapidly digested and absorbed in the intestine playing a large role in the postprandial glycaemic response. Blackcurrants are a rich source of bioactive compounds such as fibre and polyphenols compared to other berries (RP 14) has a significantly higher antioxidant capacity (Jaroslawska et al. 2016). Dietary polyphenols modulate glucose metabolism and nutrient availability by influencing the activities of digestive enzymes (Boath et al. 2012). Dietary fibre is indigestible in the small intestine and fermented by intestinal microbiota in the colon to produce short-chain fatty acids such as acetate, butyrate and propionate which have been linked to the regulation of serum cholesterol and reduced risk of cancer (Queenan et al. 2007; Buil-Cosiales et al. 2016). Various bioactive compounds have been used in food preparation to reduce the energy content and improve the colour, texture and nutritional characteristics of the final product (Brennan & Samyue 2004).

This study investigated the starch digestibility, as a predictor of glycaemic response, as well as total phenolic and antioxidant capacities of the newly developed model food containing a complex mixture of whole grains and fruit cookies. The physical properties of the model food were also analysed.

4.2. Materials and methods

4.2.1. Model food

Cookies were prepared as described in section 3.3.

4.2.2. Geometry

The geometry was analysed as described in section 3.4.

4.2.3. Colour

Colour was analysed as described in section 3.4.

4.2.4. Moisture

Moisture was determined as described in section 3.5.

4.2.5. Texture

Texture was analysed as described in section 3.6.

4.2.6. *In-vitro* carbohydrate digestibility and glycaemic response

In-vitro carbohydrate digestibility was analysed as described in section 3.7.1.

4.2.7. Determination of total phenolic content

Total phenolic compounds (TPC) was analysed as described in section 3.8.

4.2.8. Determination of antioxidant capacity by DPPH

The scavenging capacity of the samples was determined using DPPH as described in section 3.9.1.

4.2.9. Determination of antioxidant capacity by ORAC

Total antioxidant capacity by ORAC (Oxygen radical absorbance capacity) was analysed as described in section 3.9.2.

4.2.10. Statistical analysis

Statistical analysis was carried out as described in section 3.24.

4.3. Result and discussion

4.3.1. Physical properties of model food cookies

The weight loss, height and diameter of cookies are shown in Table-4.1. Increasing the proportion of blackcurrant powder in the formulation significantly ($p < 0.05$) decreased the water loss in wholemeal barley flour (9.02 % to 8.36 %) and wholemeal oat flour (10.09 % to 8.98 %) cookies compared to the control wholemeal wheat cookie group (Table-2). Water loss of the cookies ranged from 10.08 % to 8.36 % during cooking time. Diameters of cookies were influenced significantly ($p < 0.05$) with the incorporation of blackcurrant. The highest diameter (25.27 % increased) was observed in wholemeal oat cookies and the lowest diameter (0.52 % increased) was observed in wholemeal wheat cookies. Cookie expansion factor is influenced by the acid-base reaction of sodium bicarbonate (baking powder) and fat, causing bubbles in the wet dough to expand the volume and also dependent on the viscosity of the dough (Chung et al. 2014). Water absorption capacity or water holding capacity (wholemeal wheat flour 93.8 g/100 g, wholemeal oat flour 158.2 g/100 g) influences the expansion volume (Inglett et al. 2016). As the substitution level of the blackcurrant increased, the cookie diameter decreased ($p < 0.05$) in wholemeal oat and barley flour cookies, possibly due to the higher mineral content (e.g., calcium 170 mg/100g, magnesium 82 mg/100g)of blackcurrant powder that reacted with flour protein (12-13 %) resulted in a reduction in water holding capacity (Inglett et al. 2016; Perona 2017). Higher absorption capacity of wholemeal wheat cookies during the mixing of the dough resulted in a lower expansion. This was because the available water in this system was not sufficient to dissolve sugar during the baking time, thus increasing the viscosity and resulting in a lower expansion (Becker et al. 2014). Cookies height was also significantly influenced ($p < 0.05$) with the addition of blackcurrant powder.

Table 4.1. Changes after baking: Weight loss, increased height (%) and diameter (%)

Sample	Weight loss (%)	Height (%)	Diameter (%)
Oat cookie control	10.09 ± 0.07 ^a	52.78 ± 2.01 ^b	25.27 ± 1.50 ^a
Oat + 5% Blackcurrant	10.12 ± 0.15 ^a	57.94 ± 1.29 ^{ab}	17.29 ± 0.20 ^b
Oat + 10% Blackcurrant	9.44 ± 0.20 ^a	58.06 ± 3.71 ^{ab}	14.40 ± 0.06 ^c
Oat + 15% Blackcurrant	8.98 ± 0.42 ^a	61.39 ± 4.42 ^a	10.75 ± 0.34 ^d
Barley cookie control	9.02 ± 0.07 ^a	101.22 ± 2.31 ^a	7.18 ± 1.10 ^a
Barley + 5% Blackcurrant	8.73 ± 0.28 ^a	88.83 ± 3.91 ^b	5.50 ± 0.61 ^{ab}
Barley + 10% Blackcurrant	8.45 ± 0.37 ^a	80.22 ± 3.54 ^c	4.88 ± 0.65 ^b
Barley + 15% Blackcurrant	8.36 ± 0.40 ^a	70.50 ± 2.20 ^d	4.30 ± 0.89 ^b
Wheat cookie control	8.38 ± 0.18 ^b	88.11 ± 2.08 ^a	0.52 ± 0.49 ^c
Wheat + 5% Blackcurrant	9.47 ± 0.20 ^a	77.41 ± 0.58 ^b	1.87 ± 0.29 ^b
Wheat + 10% Blackcurrant	9.42 ± 0.43 ^a	71.96 ± 2.13 ^c	3.33 ± 0.41 ^a
Wheat + 15% Blackcurrant	9.68 ± 0.34 ^a	59.50 ± 1.04 ^d	1.43 ± 0.03 ^{bc}

Results are presented as the mean value ± standard deviation, $n = 3$; Data in the same columns means with the different subscript are significantly different ($p < 0.05$) within same flour cookie group.

Colour parameters L^* , a^* , b^* and ΔE values differed significantly ($p < 0.05$) among all three different sample groups. The value L^* indicates lightness ranging from 100 (white) to 0 (black). The value a^* indicates redness (positive value) and greenness (negative value). The value b^* indicates yellowness (positive value) and blueness (negative value). The value ΔE (colour difference, Delta-E) represents the colour difference within the cookie group. Substituting blackcurrant with all three control groups significantly influenced ($p < 0.05$) the surface and ground colour of the cookies (Table – 4.2, 4.3). Cookies with added blackcurrant were dark in colour compared with the control (lower L^* value). The higher a^* value indicates redness of cookies, and lower b^* value indicates lower yellowness by adding various amounts of blackcurrant. Starch dextrinization and caramelization are induced by heating also affect the cookie colour (Chevallier et al. 2000). However, as the original pigment of blackcurrant powder, degree of substitution of the blackcurrant increased, the brightness of the cookies decreased and redness increased (Table 4.2 and 4.3).

Table 4.2. The colour profile of ground cookies

Sample	<i>L</i> *	<i>a</i> *	<i>b</i> *	ΔE
Oat cookie control	84.72 ± 0.10 ^a	30.86 ± 0.09 ^d	51.82 ± 0.02 ^a	99.36 ± 0.07 ^a
Oat + 5% Blackcurrant	53.92 ± 0.04 ^b	21.16 ± 0.02 ^c	17.79 ± 0.01 ^b	57.39 ± 0.04 ^b
Oat + 10% Blackcurrant	45.77 ± 0.10 ^c	16.91 ± 0.03 ^b	14.06 ± 0.03 ^c	50.78 ± 0.11 ^c
Oat + 15% Blackcurrant	31.47 ± 0.20 ^d	17.21 ± 0.11 ^a	11.16 ± 0.05 ^d	37.57 ± 0.23 ^d
Barley cookie control	84.30 ± 0.30 ^a	3.84 ± 0.17 ^d	46.08 ± 0.07 ^a	96.15 ± 0.29 ^a
Barley + 5% Blackcurrant	72.92 ± 0.42 ^b	12.85 ± 0.35 ^c	37.26 ± 0.12 ^d	74.14 ± 0.36 ^b
Barley + 10% Blackcurrant	54.20 ± 0.11 ^c	18.35 ± 0.070 ^b	18.87 ± 0.02 ^b	60.26 ± 0.13 ^c
Barley + 15% Blackcurrant	42.71 ± 0.44 ^d	21.11 ± 0.11 ^a	15.49 ± 0.12 ^c	50.10 ± 0.46 ^d
Wheat cookie control	91.72 ± 0.17 ^a	2.13 ± 0.08 ^c	26.09 ± 0.10 ^a	95.38 ± 0.14 ^a
Wheat + 5% Blackcurrant	70.71 ± 0.60 ^b	9.55 ± 0.23 ^b	4.94 ± 0.11 ^b	71.53 ± 0.56 ^b
Wheat + 10% Blackcurrant	56.39 ± 0.89 ^c	20.16 ± 0.39 ^a	3.02 ± 0.08 ^c	59.96 ± 0.71 ^c
Wheat + 15% Blackcurrant	56.92 ± 0.10 ^c	19.61 ± 0.34 ^a	3.06 ± 0.43 ^c	60.28 ± 0.07 ^c

Results are presented as the mean value ± standard deviation, $n = 3$; Data in the same columns means with the different subscript are significantly different ($p < 0.05$) within same flour cookie group.

Table 4.3. The colour profile of the surface of the cookie

Sample	<i>L</i> *	<i>a</i> *	<i>b</i> *	ΔE
Oat cookie control	89.12 ± 0.14 ^a	-4.99 ± 0.19 ^b	34.02 ± 0.11 ^a	95.52 ± 0.14 ^a
Oat + 5% Blackcurrant	79.59 ± 0.18 ^b	-3.38 ± 0.15 ^a	21.59 ± 0.08 ^b	82.53 ± 0.14 ^b
Oat + 10% Blackcurrant	77.62 ± 0.34 ^c	-2.82 ± 0.27 ^a	20.85 ± 0.05 ^c	80.42 ± 0.33 ^c
Oat + 15% Blackcurrant	76.45 ± 0.16 ^d	-3.24 ± 0.25 ^a	20.48 ± 0.06 ^d	79.21 ± 0.16 ^d
Barley cookie control	94.04 ± 0.06 ^a	-10.34 ± 0.23 ^c	35.14 ± 0.01 ^a	100.92 ± 0.07 ^a
Barley + 5% Blackcurrant	82.93 ± 0.05 ^b	-3.31 ± 0.18 ^b	23.71 ± 0.72 ^b	86.32 ± 0.17 ^b
Barley + 10% Blackcurrant	78.90 ± 0.14 ^c	-2.13 ± 0.14 ^a	21.93 ± 0.12 ^c	81.92 ± 0.17 ^c
Barley + 15% Blackcurrant	76.78 ± 0.08 ^d	-3.72 ± 0.08 ^b	21.46 ± 0.04 ^c	79.81 ± 0.08 ^d
Wheat cookie control	88.40 ± 0.17 ^a	-4.44 ± 0.15 ^c	31.25 ± 0.12 ^a	93.87 ± 0.19 ^a
Wheat + 5% Blackcurrant	80.32 ± 0.08 ^b	-3.68 ± 0.06 ^b	22.59 ± 0.30 ^b	83.51 ± 0.12 ^b
Wheat + 10% Blackcurrant	77.22 ± 0.13 ^c	-3.00 ± 0.15 ^a	20.98 ± 0.13 ^c	80.07 ± 0.15 ^c
Wheat + 15% Blackcurrant	76.34 ± 0.14 ^d	-3.53 ± 0.17 ^b	20.62 ± 0.07 ^c	79.16 ± 0.14 ^d

Results are presented as the mean value ± standard deviation, $n = 3$; Data in the same columns means with the different subscript are significantly different ($p < 0.05$) within same flour cookie group.

Moisture content of the cookies increased with increasing proportion of blackcurrant powder in the formulation of wholemeal oat (4.63 % to 6.58 %) and wheat (7.76 % to 8.91 %) cookie group, but decreased in wholemeal barley (9.76 % to 7.44 %) cookie group, this can be explained by differences in water holding capacity (Torbica et al. 2012). A textural property is one of the important factors contributing to the cookie quality. As the level of substitution of blackcurrant powder increased, the textural property of the cookies significantly changed ($P > 0.05$) as shown in Table 4.4. This data indicates that the cookies tended to become harder as the level of blackcurrant powder increased

with the wholemeal barley (4.85 to 7.64 kg) and oat (6.85 to 10.61 kg) flour, and become softer with the wholemeal wheat (10.48 to 7.26 kg) flour. These changes may be due to changes in the interaction of starch and protein and their hydrogen bonding (Brennan & Samyue 2004; Mais & Brennan 2008). Moreover, the moisture content of the cookies might have impact on cookies textural property.

Table 4.4. After bake cookie moisture content and hardness (kg)

Sample	Moisture content (%)	Hardness (Kg)
Oat cookie control	4.63 ± 0.01 ^d	6.85 ± 0.42 ^b
Oat + 5% Blackcurrant	4.87 ± 0.01 ^c	7.98 ± 0.81 ^b
Oat + 10% Blackcurrant	5.88 ± 0.04 ^b	10.10 ± 0.67 ^a
Oat + 15% Blackcurrant	6.58 ± 0.01 ^a	10.61 ± 0.56 ^a
Barley cookie control	9.76 ± 0.01 ^a	4.85 ± 0.13 ^d
Barley + 5% Blackcurrant	7.32 ± 0.07 ^b	5.80 ± 0.12 ^c
Barley + 10% Blackcurrant	7.16 ± 0.12 ^b	6.70 ± 0.24 ^b
Barley + 15% Blackcurrant	7.44 ± 0.11 ^b	7.64 ± 0.17 ^a
Wheat cookie control	7.76 ± 0.03 ^b	10.48 ± 1.03 ^a
Wheat + 5% Blackcurrant	7.83 ± 0.14 ^b	8.93 ± 0.35 ^{ab}
Wheat + 10% Blackcurrant	8.70 ± 0.05 ^a	8.53 ± 1.14 ^b
Wheat + 15% Blackcurrant	8.91 ± 0.04 ^a	7.26 ± 0.60 ^b

Results are presented as the mean value ± standard deviation, $n = 3$; Data in the same columns means with the different subscript are significantly different ($p < 0.05$) within same flour cookie group.

4.3.2. Total phenolic content (TPC) of model food

The TPC contents are presented in Table 4.5. The total amount of phenolics were significantly ($p < 0.05$) different and linearly increased with the increased proportion of blackcurrant in all three groups model food cookies. The higher amount of TPC were observed in B15BC (3.87 mg GAE g⁻¹), O15BC (3.53 mg GAE g⁻¹) and W15BC (3.29 mg GAE g⁻¹) which is six fold higher compare with control group, whereas the range of TPC content (mg/g) in blackcurrant 14-18 (Kahkonen et al. 2001). Similarly, consistent results also reported in other literatures that higher phenolic content exhibited in barley then oat, followed by wheat (Zielinski & Kozłowska 2000; Zilic et al. 2011). So, TPC significantly increased ($p < 0.05$) in wholemeal flour cookies with increase proportion of blackcurrant, due to higher total phenolic content of blackcurrant.

Table 4.5. Total phenolic content and antioxidant capacity

Samples	TPC (mg GAE g ⁻¹ sample)	DPPH (μmol TE g ⁻¹ sample)	ORAC (mmol TE g ⁻¹ sample)
Wheat cookie control	0.53 ± 0.02 ^d	0.58 ± 0.01 ^d	0.06 ± 0.01 ^c
Wheat + 5% Blackcurrant	1.43 ± 0.01 ^c	0.92 ± 0.02 ^c	0.08 ± 0.01 ^b
Wheat + 10% Blackcurrant	2.30 ± 0.02 ^b	1.83 ± 0.02 ^b	0.09 ± 0.01 ^{ab}
Wheat + 15% Blackcurrant	3.29 ± 0.02 ^a	2.01 ± 0.03 ^a	0.11 ± 0.01 ^a
Barley cookie control	0.67 ± 0.03 ^d	0.29 ± 0.04 ^c	0.04 ± 0.00 ^d
Barley + 5% Blackcurrant	1.98 ± 0.01 ^c	0.88 ± 0.09 ^b	0.05 ± 0.01 ^c
Barley + 10% Blackcurrant	2.90 ± 0.02 ^b	1.82 ± 0.05 ^a	0.07 ± 0.00 ^b
Barley + 15% Blackcurrant	3.87 ± 0.02 ^a	1.90 ± 0.03 ^a	0.10 ± 0.00 ^a
Oat cookie control	0.66 ± 0.01 ^d	0.43 ± 0.01 ^c	0.05 ± 0.01 ^c
Oat + 5% Blackcurrant	1.56 ± 0.01 ^c	0.81 ± 0.01 ^b	0.06 ± 0.00 ^c
Oat + 10% Blackcurrant	2.48 ± 0.01 ^b	0.89 ± 0.03 ^a	0.08 ± 0.00 ^b
Oat + 15% Blackcurrant	3.53 ± 0.01 ^a	1.12 ± 0.01 ^a	0.10 ± 0.00 ^a

Results are presented as the mean value ± standard deviation, $n = 3$; Data in the same columns means with the different subscript are significantly different ($p < 0.05$) within same flour cookie group.

4.3.3. Antioxidant capacity

It is well established that the potential health benefits of bioactive compounds result mainly from their antioxidant activities. Generally, the presence of higher phenolic compounds of the sample can be correlated with the higher antioxidant capacity. Notably, increasing the level of blackcurrant powder in the formulation significantly ($p < 0.05$) increased the ability to scavenge DPPH free radical, which is correlated with the TPC content of the model food. Antioxidant capacity also depends on the other bioactive compounds, such as ascorbic acid, and type of compounds present in the sample (Palafox-Carlos et al. 2012). Antioxidant capacity of blackcurrant containing cookies was higher than that of control cookies. This could be attributed to the higher total phenolic content of blackcurrant. The DPPH (%) scavenging activity and Trolox equivalent (TE) are shown in Table 4.5. The enrichment of cookie formulation with 5 %, 10 % and 15 % blackcurrant with the flour, the observed DPPH radical scavenging activity were significantly ($p < 0.05$) higher in W15BC (2.01 μmol TE g⁻¹), B15BC (1.90 μmol TE g⁻¹) and O15BC (1.12 μmol TE g⁻¹) than the control group, which scavenged 47.05 % (W15BC), 33.56 % (B15BC) and 16.22 % (O15BC) of the DPPH radical. The blackcurrant powder enriched cookies capacity to scavenge DPPH is mainly attributed to the presence of blackcurrant powder, which was confirmed as a potent DPPH scavenger.

The positive association was exhibited with the total phenolic content and two antioxidant capacity (DPPH and ORAC) assays, and all data exhibited linear relationship. Blackcurrant enriched cookies also exhibited higher ORAC (oxygen radical absorbance capacity) values as enhancement of total phenolic content. ORAC assays demonstrated that blackcurrant enriched cookies more effective in neutralizing peroxy radicals than the control cookies, which was expressed as Trolox equivalent (shown in Table 4.6). ORAC values exhibited significantly ($p < 0.05$) higher in W15BC (0.11 mmol TE g⁻¹), B15BC (0.10 mmol TE g⁻¹) and O15BC (0.10 mmol TE g⁻¹) in comparison with the control groups. These results suggest that cookies with added blackcurrant enhanced nutraceutical properties of cookies and potential health benefit, as exploited data yet to be substantiated.

Table 4.6. Total phenolic content and antioxidant capacity

Sample	TPC mg GAE g ⁻¹	DPPH μ mol TE g ⁻¹	ORAC mmolTE g ⁻¹
Sample	Sample	sample	sample
Wheat flour	1.12 \pm 0.03 ^c	0.81 \pm 0.01 ^c	0.10 \pm 0.00 ^b
Barley flour	1.58 \pm 0.03 ^b	1.57 \pm 0.04 ^b	0.10 \pm 0.01 ^b
Oat flour	0.84 \pm 0.01 ^d	0.79 \pm 0.01 ^c	0.11 \pm 0.00 ^b
Blackcurrant powder	12.73 \pm 0.05 ^a	1.57 \pm 0.03 ^a	7.19 \pm 0.06 ^a

Results are presented as the mean value \pm standard deviation, $n = 3$; Data in the same columns means with the different subscript are significantly different ($p < 0.05$) within same flour cookie group.

4.3.4. *In-vitro* starch digestibility and AUC

The rate of glucose release during digestion is related to the starch degradation and subsequently glycaemic response of an individual. Glucose release was calculated as the reducing sugars hydrolysed from starch by digestive enzymes (Figure 4.1). All cookie samples showed that the rate of sugar release rapidly increased in the first 20 minutes of the digestion including control. Therefore, increased amount of blackcurrant powder in the model food slowdown the rate of sugar release significantly ($p < 0.05$) during the digestion process. Blackcurrant powder could be responsible for the decreased rate of sugar released, and hence the rate of starch degradation of cookies. Blackcurrants contain high amounts of polyphenol and are a rich source of antioxidant (Kahkonen et al. 2003), which are effective inhibitors of digestive enzymes (Matsiu et al. 2002). The inhibition of enzymes activity may be attributed to the interactions between enzymes and phenolic substances, for example complex formation of proanthocyanidins and α -amylase (Grussu et al. 2011; Barros et al.

2012) and/or starch-phenolic non-covalent interactions (Bordenave et al. 2014) affect starch retrogradation. Generally, phenolic compounds could be readily oxidized in alkaline solutions and produce reactive species, which interact with the free amino group of protein. As a result of these reactions of phenolic compounds and proteins changes the physicochemical properties for example solubility, molecular weight, thermodynamic parameter and secondary/tertiary structure of model food. On the other hand, the non-covalent interaction between starch and phenolic compounds involve hydrogen bond, hydrophobic and ionic interactions (Bordenave et al. 2014; Soong et al. 2014) form a complex and affect starch degradation.

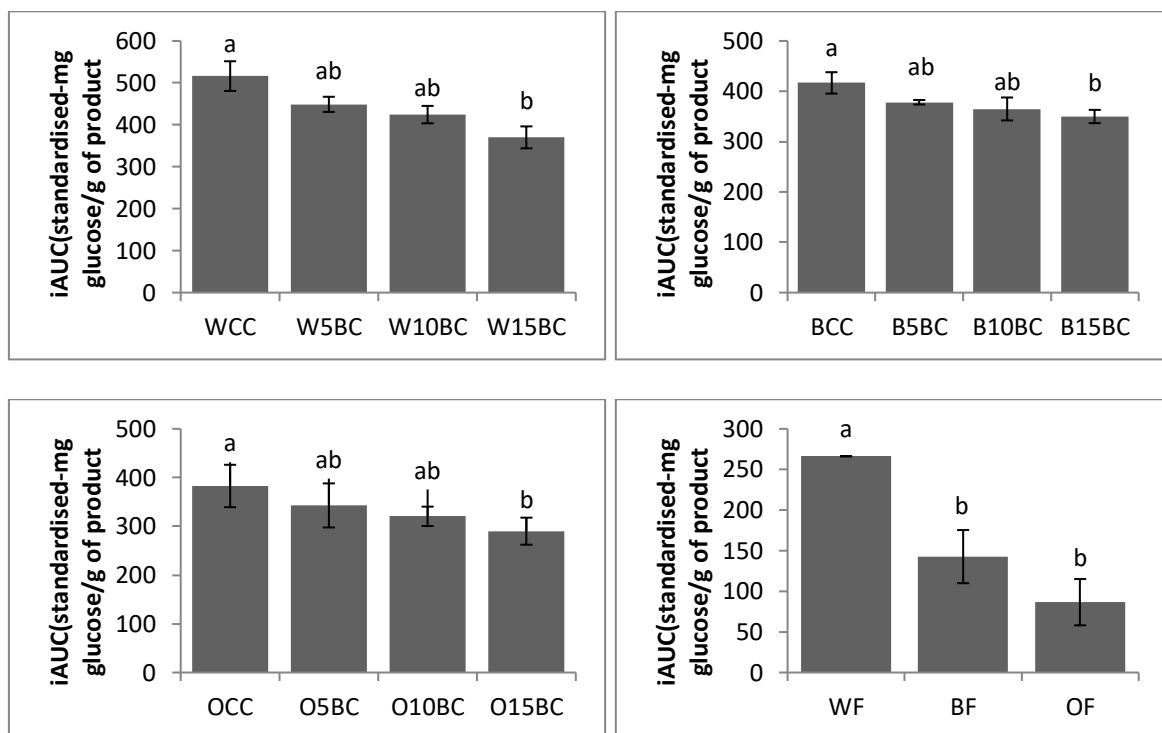


Figure 4.1. Reducing sugar release over 120 min (expressed as mg reducing sugar released / g available starch) digestion of wholemeal (a) wheat, (b) barley (c) oat cookies and (d) Wholemeal flour with degree of substitution of blackcurrant.

Abbreviation: (a) WCC – Wholemeal wheat cookie control; W5BC – Wholemeal wheat + 5% blackcurrant; W10BC - Wholemeal wheat + 10% blackcurrant; W15BC - Wholemeal wheat + 5% blackcurrant; (b) BCC- Wholemeal barley cookie control; B5BC – Wholemeal barley + 5% blackcurrant; B10BC- Wholemeal barley + 10% blackcurrant ; B15BC - Wholemeal barley + 15% blackcurrant; (c) OCC- Wholemeal oat cookie control; O5BC – Wholemeal oat + 5% blackcurrant; O10BC- Wholemeal oat + 10% blackcurrant ; O15BC - Wholemeal oat + 15% blackcurrant; (d) WF – Wholemeal wheat flour; BF – Wholemeal barley flour; OF – Wholemeal oat flour

4.4. Conclusion

The study demonstrated that the physical and chemical properties of the model food cookie were influenced by the addition of blackcurrant powder. In comparison with control sample, significant

improvement of bioactive compound was exhibited, this was associated with a significant decrease of the content of rapidly available glucose (T20) and subsequent *in-vitro* digestion (T120). Furthermore, potential glycaemic response modulated by the fraction of starch and bioactive compounds which affected carbohydrate digestion as postulated using *in-vitro* digestion model. In this study significantly higher amount of total phenolic compounds and higher values of antioxidant capacity were observed in blackcurrant enriched model food cookies. Therefore, considering the potential synergies of bioactive compounds present in blackcurrant and wholemeal flour may be another important source for the potential health benefits attributed to a diet to regulate the glycaemic response of foods.

Chapter 5

The effect of an astaxanthin-rich microalgae “*Haematococcus pluvialis*” and wholemeal flours in improving physical and functional properties of cookies

Hossain, A., M. A. Brennan, S. L. Mason, X. Guo, X. A. Zeng and C. S. Brennan (2017). "The effect of astaxanthin-rich microalgae “*Haematococcus pluvialis*” and wholemeal flours incorporation in improving the physical and functional properties of cookies." Foods 6(8): 57.

Abstract

A significant amount of bioactive compounds in a diet is important to sustain human health. In an effort to modulate glycaemic response and enhance nutritional aspects, marine-derived algal food rich in astaxanthin was used in the formulation of a model food cookie. Astaxanthin substitution by three wholemeal flours (wheat, barley and oat) demonstrated a significant reduction in the rate of glucose released during the *in-vitro* digestion and an increase in the TPC and antioxidant capacity of the model food. The significantly ($P < 0.005$) lower free glucose release was observed from cookies with 15 % astaxanthin, followed by 10 % and then 5 % astaxanthin in comparison with control cookies of each flour. The TPC, DPPH and ORAC value also notably increased with increase in astaxanthin content. The results represent the potential use of microalgae to enhance the bioactive compounds and lower the glycaemic response of wholemeal flour cookie.

5.1. Introduction

Marine algae which are more commonly known as seaweeds have been used in the human diet since 600 BC (Aguilera-Morales et al. 2005). With the growing awareness and increasing research interest regarding the role of diet in sustaining human health, nutritional research has been revealed marine algae have excellent potential health benefits due to their diverse range of nutrients and bioactive compounds (such as polysaccharides, proteins, polyunsaturated fatty acids and minerals) and

significant amounts of antioxidants (Plaza et al. 2008; Lordan et al. 2011). *Haematococcus pluvialis* is a well-known single-cell microalgal strain which is rich source of astaxanthin (10,000-40,000 mg/Kg) (Wu et al. 2014b). Several cell culture and animal studies have reported that astaxanthin is a carotenoid that has potent antioxidant activity which is 10 times higher than other carotenoids such as β -carotene, lutein, and zeaxanthin and 500 times higher than vitamin E (Miki 1991; Shimidzu et al. 1996; Naguib 2000). Carotenoids play a role in preventing or delaying degenerative diseases such as aging, cancer and atherosclerosis diseases (Riccioni et al. 2011; Barros et al. 2014; Raposo et al. 2015). So, it will be useful to develop foods containing microalgae. Whole-grains such as wheat, barley and oat make a substantial contribution to our diet. Apart from the basic nutritional elements whole-grains contain significant amount of bioactive compounds such as fibre, minerals, vitamins and phytochemicals (Brennan & Cleary 2005; Andersson et al. 2014) and may play a major role in enhancing human health such as a reduction of the risk of type 2 diabetes (Brennan 2005; Ye et al. 2012), cancer (Knudsen et al. 2014), regulation of serum cholesterol (Cho et al. 2013) and stimulation of beneficial gut microbiota (Zhou et al. 2015). There is a paucity of information regarding combining the nutritional facts of marine-based compounds and whole-grains. Therefore, the present study is the first to show the glycaemic glucose equivalents (GGE) as a predictor of glycaemic response, antioxidant capacities and physical properties of cereal and *H. pluvialis* model foods.

5.2. Materials and methods

5.2.1. Sample collection and preparation

Described in section 3.2.

5.2.2. Model food

The model food (cookies) was prepared as described in section 3.3.

5.2.3. Physical characteristics

Cookie physical characteristics (height, weight and color) was analysed as described in section 3.4.

5.2.4. Moisture

Moisture was analysed as described in section 3.5.

5.2.5. Texture

Texture was analysed as described in section 3.6.

5.2.6. Determination of total phenolic content

The content of total phenolics of samples was determined as described in section 3.8.

5.2.7. Antioxidant properties

The antioxidant capacity of the samples was measured by the DPPH as described in section 3.9.1.

Total antioxidant capacity by ORAC was determined as described in section 3.9.2.

5.2.8. *In-vitro* carbohydrate digestion (glycaemic glucose equivalent-GGE) analysis

In-vitro carbohydrate digestion was determined as described in section 3.7.1.

5.2.9. Statistical analysis

Statistical analysis was carried out as described in section 3.24.

5.3 Result and discussion

The model food was prepared with the astaxanthin powder and wholemeal flour. The effect of astaxanthin powder rational replacement on the physical properties and nutritional values of wholemeal flour cookies were analysed.

5.3.1. Physical properties of model food cookie

The physical characteristics of the model cookies are summarised in Table 5.1. The results showed a significant effect ($p < 0.05$) with addition of astaxanthin on the height, diameter and bake loss of the three flour types of cookies. As the amount of astaxanthin powder decrease (15% > 10% > 5% > 0 % astaxanthin) the weight loss, height and diameter decreased. The largest height and diameter changes were observed in cookies made from wholemeal wheat flour. This observation could be attributed to the hydrophilic nature of the ingredients (Okpala et al. 2013). The spread factor of a cookie is affected by the acid-base reaction of the ingredients (sodium bicarbonate and fat), causing bubble in the dough to expand in volume and is also dependent on the viscosity of the cookie dough (Chung et al. 2014). Physical evaluation of the cookies reported by Brennan and Samyue (2004) and Giami et al. (2005), illustrated that the spread factor is affected by the water holding capacity of the ingredients. Cookies made with wholemeal barley showed that the moisture content increased with the increasing addition of astaxanthin. The reason of this phenomenon is suggested in other papers to be the physical state of starch, protein and fibre are the key determinants of water holding capacity of the flour (Ragae & Abdel-Aal 2006; Mais & Brennan 2008; Yamsaengsung et al. 2012). The moisture content of three kinds of flour cookies significantly increased at all level of astaxanthin addition (Table 2). This can be attributed to differences in water holding capacity of the ingredients especially different flours (Inglett et al. 2015). Correspondingly, the hardness of the cookies decreased with the addition of astaxanthin (Table 5.1). The study indicated that the astaxanthin incorporated wheat and oat cookies became softer in comparison to control cookies. It suggested that water holding capacity of astaxanthin intermediary between oat and barley flour. The

differences in swelling behaviour of the starch granules resulted in cookies with different textural properties, increased protein content and the interaction of starch and protein and their hydrogen bonding during dough development (Mais & Brennan 2008; Kweon et al. 2011).

Table 5.1. Physical characteristics (after baking: changes in height (%), diameter (%) and weight loss (%); moisture content (%) and hardness (Kg) of the model cookies)

Sample	Increase in height (%)	Increase in diameter (%)	Weight loss (%)	Moisture content (%)	Hardness (kg)
WCC	94.39 ± 3.06 ^a	3.93 ± 0.22 ^a	9.71 ± 0.04 ^a	7.50 ± 0.11 ^c	9.26 ± 0.13 ^a
W5A	71.44 ± 8.39 ^b	2.96 ± 1.13 ^{ab}	9.63 ± 0.02 ^{ab}	7.83 ± 0.01 ^b	7.79 ± 0.16 ^b
W10A	59.39 ± 3.06 ^{bc}	2.27 ± 0.21 ^{ab}	9.48 ± 0.12 ^{ab}	7.91 ± 0.07 ^b	7.35 ± 0.58 ^b
W15A	52.94 ± 0.75 ^c	1.15 ± 0.92 ^b	9.44 ± 0.12 ^b	8.21 ± 0.03 ^a	7.06 ± 0.48 ^b
BCC	94.33 ± 6.78 ^a	5.23 ± 1.16 ^a	10.31 ± 0.11 ^a	7.74 ± 0.02 ^d	4.12 ± 0.12 ^c
B5A	83.50 ± 1.04 ^{ab}	4.76 ± 0.44 ^a	10.41 ± 0.11 ^a	7.79 ± 0.02 ^c	4.98 ± 0.20 ^b
B10A	74.61 ± 2.91 ^{bc}	3.67 ± 0.73 ^{ab}	10.46 ± 0.20 ^a	7.90 ± 0.01 ^b	5.26 ± 0.22 ^b
B15A	65.50 ± 4.84 ^c	2.72 ± 0.31 ^b	10.67 ± 0.28 ^a	8.17 ± 0.02 ^a	6.21 ± 0.10 ^a
OCC	70.94 ± 0.91 ^a	23.20 ± 0.25 ^a	11.54 ± 0.17 ^a	5.55 ± 0.05 ^d	7.57 ± 0.05 ^a
O5A	67.94 ± 2.46 ^{ab}	12.81 ± 0.49 ^b	11.14 ± 0.23 ^{ab}	6.09 ± 0.04 ^c	7.23 ± 0.14 ^{ab}
O10A	64.55 ± 0.25 ^b	7.80 ± 0.05 ^c	10.63 ± 0.22 ^{bc}	6.66 ± 0.04 ^b	7.02 ± 0.12 ^b
O15A	55.55 ± 0.91 ^c	3.62 ± 0.14 ^d	10.23 ± 0.23 ^c	7.12 ± 0.09 ^a	6.16 ± .023 ^c

Data are presented as mean ± standard deviation, n = 3; Means within same columns for same flour cookie group do not share the same superscript are significantly different ($P < 0.05$). W- Wheat, B-Barley, O-Oat, CC-Cookie Control, A-Astaxanthin (5 %, 10 % or 15 %)

The colour profile (L, a, b and colour difference ΔE) of the cookie samples (surface and ground) is summarised in Table 5.2. The addition of astaxanthin to the three types of flour cookies significantly ($p < 0.05$) decreased the lightness (L^*) of all cookie groups. Addition of astaxanthin significantly ($P < 0.05$) decreased redness (a^*) and increased blueness (b^*) of the wholemeal wheat cookie group, and increased redness (a^*) and decreased blueness (b^*) of the wholemeal barley and oat cookie groups. There was a significant colour change as illustrated by the ΔE value of the three kind of flour cookies in the following order: control cookies > 5 % astaxanthin > 10 % astaxanthin > 15 % astaxanthin cookies. The reason for the cookie colour is due to the pigment of astaxanthin powder as the level of substitution increased, a^* values moved from greenness (-ve) towards redness (+ve). However, the reaction between reducing sugars and amino acids (Maillard reaction; starch dextrinization and caramelization) which is induced by heating during baking time also enhances darkness the cookie colour (Chevallier et al. 2000) as reflected in colour difference (Table 5.2).

Table 5.2. The colour profile of the cookies

A. Surface Cookie colour				
Sample	L*	a*	b*	ΔE
WCC	90.40 ± 0.42 ^a	-5.79 ± 0.35 ^a	33.05 ± 0.09 ^a	96.43 ± 0.44 ^a
W5A	84.14 ± 0.26 ^b	-7.37 ± 0.08 ^b	29.16 ± 0.23 ^b	89.36 ± 0.32 ^b
W10A	82.20 ± 0.10 ^c	-8.72 ± 0.20 ^c	27.42 ± 0.06 ^c	87.09 ± 0.13 ^c
W15A	81.42 ± 0.32 ^c	-8.27 ± 0.08 ^{±c}	26.45 ± 0.34 ^d	86.01 ± 0.40 ^d
BCC	94.43 ± 0.45 ^a	-8.16 ± 0.76 ^a	34.55 ± 0.27 ^a	100.89 ± 0.58 ^a
B5A	86.97 ± 0.19 ^b	-9.25 ± 0.04 ^b	32.16 ± 0.07 ^b	93.19 ± 0.20 ^b
B10A	84.77 ± 0.23 ^c	-7.88 ± 0.25 ^a	30.06 ± 0.15 ^c	90.29 ± 0.27 ^c
B15A	83.10 ± 0.14 ^d	-7.83 ± 0.01 ^a	27.95 ± 0.14 ^d	88.02 ± 0.18 ^d
OCC	91.31 ± 0.69 ^a	-5.64 ± 0.34 ^a	35.24 ± 0.15 ^a	98.04 ± 0.71 ^a
O5A	84.19 ± 0.14 ^b	-7.63 ± 0.34 ^b	30.64 ± 0.15 ^b	89.91 ± 0.20 ^b
O10A	82.21 ± 0.13 ^c	-7.87 ± 0.13 ^b	28.26 ± 0.11 ^c	87.31 ± 0.17 ^c
O15A	80.77 ± 0.15 ^d	-8.17 ± 0.13 ^b	26.56 ± 0.19 ^d	85.39 ± 0.22 ^d
B. Ground Cookie colour				
Sample	L*	a*	b*	ΔE
WCC	87.20 ± 0.20 ^a	-0.32 ± 0.03 ^a	45.33 ± 0.10 ^a	97.55 ± 0.30 ^a
W5A	77.82 ± 0.06 ^b	-6.32 ± 0.04 ^c	43.72 ± 0.26 ^b	90.29 ± 0.01 ^b
W10A	75.42 ± 0.22 ^c	-6.98 ± 0.03 ^d	38.31 ± 0.15 ^c	84.88 ± 0.13 ^c
W15A	69.10 ± 0.30 ^d	-6.20 ± 0.02 ^b	34.13 ± 0.12 ^d	77.32 ± 0.22 ^d
BCC	95.13 ± 0.07 ^a	-13.41 ± 0.21 ^c	43.14 ± 0.41 ^a	105.32 ± 0.13 ^a
B5A	82.96 ± 0.62 ^b	-5.86 ± 0.23 ^a	43.95 ± 0.65 ^a	94.06 ± 0.30 ^b
B10A	74.46 ± 0.63 ^c	-6.56 ± 0.12 ^b	44.02 ± 0.09 ^a	86.74 ± 0.56 ^c
B15A	71.13 ± 0.77 ^d	-6.04 ± 0.38 ^{ab}	39.85 ± 1.38 ^b	81.76 ± 0.24 ^d
OCC	93.15 ± 0.59 ^a	-9.32 ± 0.134 ^b	49.41 ± 1.35 ^a	105.86 ± 0.02 ^a
O5A	85.92 ± 0.27 ^b	-8.36 ± 0.09 ^{ab}	39.58 ± 0.21 ^{bc}	94.97 ± 0.16 ^b
O10A	73.24 ± 0.36 ^c	-7.33 ± 0.03 ^a	36.04 ± 0.07 ^c	81.96 ± 0.29 ^c
O15A	71.19 ± 0.47 ^d	-6.99 ± 0.57 ^a	41.34 ± 2.68 ^b	82.64 ± 0.97 ^c

Data are presented as mean ± standard deviation, n = 3; Means within same columns for same flour cookie group do not share the same superscript are significantly different ($P < 0.05$). W- Wheat, B-Barley, O-Oat, CC- Cookie Control, A-Astaxanthin (5 %, 10 % or 15 %)

5.3.2. Total phenolic content (TPC) and antioxidant activity of model food cookie

The phenolic content and DPPH radical scavenging activity and ORAC of model cookies are summarised in Table 5.3. In view of the fact that the phenolic content of the samples is proportionally associated with the antioxidant activity. TPC was significantly ($P < 0.05$) increased with the replacement of astaxanthin powder 5 > 10 > 15 % respectively with the wholemeal wheat, barley and oat flour. The phenomenon is due to the high phenolics in astaxanthin (10,000 – 40,000 mg/kg) (Spiller & Dewell 2003). Similarly, consistent results have also been reported by (Sharma & Gujral

2014) that wheat flour has less phenolics as compared to barley and oat flour. Increasing the level of astaxanthin in cookies resulted in a significant increase antioxidant activity (Table 5.3) towards DPPH scavenging activity. This could be due to the astaxanthin of microalgae, which have been reported 10 times stronger than the other carotenoids (Miki 1991). ORAC values linearly increased (mmol TE g⁻¹ sample) as astaxanthin powder increased in the formulation. However, the antioxidant capacity of a compound is dependent upon type of antioxidant compounds, sensitivity and reaction media (Huang 2005).

Table 5.3. Total phenolic content and antioxidant capacity

Sample	TPC (mg GAE g ⁻¹ sample)	DPPH (μmol g ⁻¹ sample)	ORAC (mmol TE g ⁻¹ sample)
WCC	0.59 ± 0.01 ^d	0.54 ± 0.01 ^d	0.09 ± 0.001 ^b
W5A	0.80 ± 0.01 ^c	0.95 ± 0.03 ^c	0.11 ± 0.001 ^a
W10A	0.95 ± 0.01 ^b	1.10 ± 0.01 ^b	0.12 ± 0.001 ^a
W15A	1.14 ± 0.01 ^a	1.26 ± 0.03 ^a	0.12 ± 0.004 ^a
BCC	0.63 ± 0.01 ^c	1.36 ± 0.01 ^d	0.08 ± 0.003 ^b
B5A	0.95 ± 0.02 ^b	1.69 ± 0.02 ^c	0.09 ± 0.002 ^a
B10A	1.15 ± 0.09 ^a	1.74 ± 0.01 ^b	0.09 ± 0.002 ^a
B15A	1.27 ± 0.01 ^a	1.79 ± 0.01 ^a	0.10 ± 0.002 ^a
OCC	0.87 ± 0.01 ^d	1.13 ± 0.01 ^d	0.08 ± 0.001 ^c
O5A	1.03 ± 0.01 ^c	1.22 ± 0.01 ^c	0.10 ± 0.002 ^b
O10A	1.28 ± 0.01 ^b	1.34 ± 0.01 ^b	0.10 ± 0.001 ^a
O15A	1.44 ± 0.01 ^a	1.46 ± 0.01 ^a	0.11 ± 0.001 ^a

Data are presented as mean ± standard deviation, n = 3; Means within same columns for same flour cookie group do not share the same superscript are significantly different ($P < 0.05$). W- Wheat, B-Barley, O-Oat, CC-Cookie Control, A-Astaxanthin (5 %, 10 % or 15 %)

5.3.3. Glycaemic glucose equivalent (GGE) analysis and area under the curve (AUC)

Figure 5.1 illustrates the *in-vitro* digestion of the model food, calculated as amount of reducing sugar released by digestive enzymes over 120 min. All the samples demonstrated the impact of the substitution of astaxanthin in the following order (5 % > 10 % > 15 %) and significantly slowest the rate of reducing sugar released (Fig. 1, calculated as mg glucose g⁻¹ sample of incremental area under the curve-iAUC) as compare with the control cookie. The cookie with 15% astaxanthin showed the lowest absolute glucose release followed by 10 % and then 5% astaxanthin inclusion. Astaxanthin powder could be related to the decrease the rate of sugar released due to higher phenolic content and antioxidant activity (Naguib 2000), that can impair enzymes activity during the digestion (Matsiu

et al. 2002). The interaction between phenolics and digestive enzymes (Paliwal et al. 2015) and non-covalent interactions of starch-phenolics affect starch degradation (Bordenave et al. 2014; Soong et al. 2014). Moreover, the rate of sugar release decreased due to the non-starchy network (mainly fibre and protein in the system) which is entrapping starch granules and act as a physical barrier resulting limit enzyme accessibility (Wolter et al. 2014; Foschia et al. 2015).

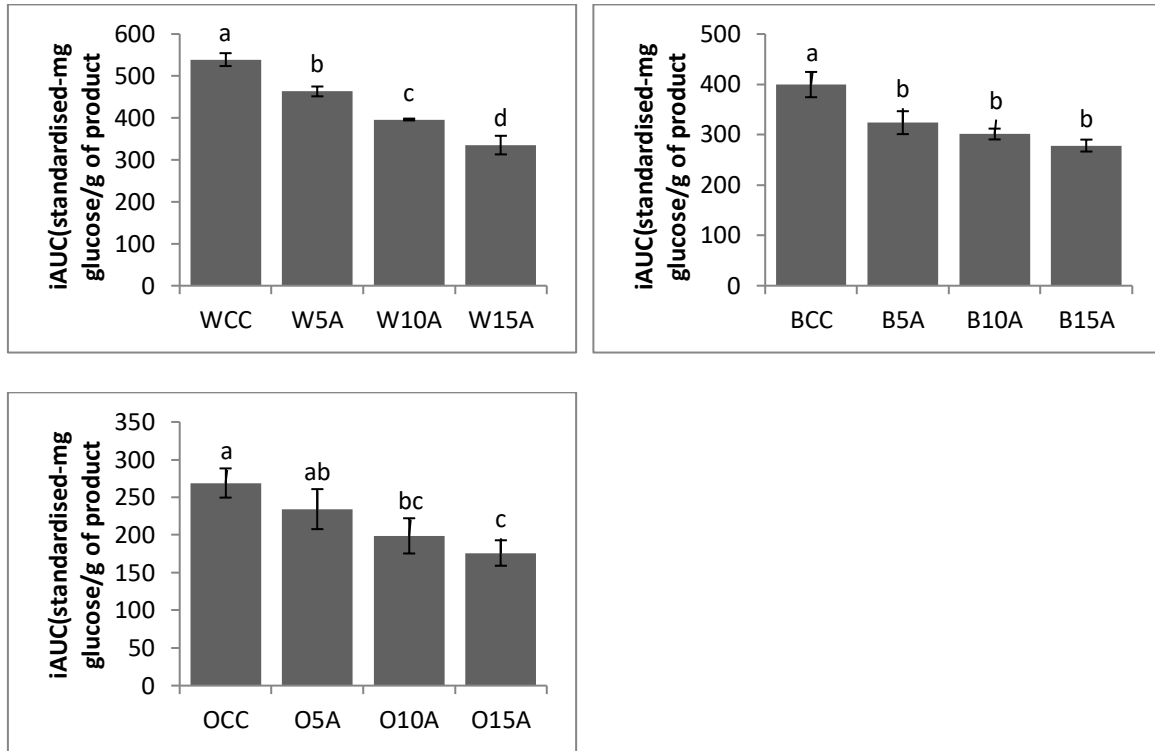


Figure 5.1. Reducing sugar released (mg/ g sample) after 120 min digestion of three kinds of wholemeal flour cookies with astaxanthin substitution;

Abbreviation: iAUC = incremental area under the curve; WCC –wheat cookie control; W5A –wheat + 5% astaxanthin cookie; W10A - wheat + 10% astaxanthin cookie; W15A - wheat + 5% astaxanthin cookie; BCC- barley cookie control; B5A –barley + 5% astaxanthin cookie; B10A- barley + 10% astaxanthin cookie; B15A - barley + 15% astaxanthin cookie; OCC- oat cookie control; O5A –oat + 5% astaxanthin cookie; O10A- oat + 10% astaxanthin cookie; O15A - oat + 15% astaxanthin cookie;

5.4. Conclusion

Qualitative differences of carbohydrate of starchy foods in combination with new natural ingredients with potential biological activity have shown great possibilities food with enhanced nutritional qualities. In particular, *in-vitro* digestion (GGE analysis) of the model food was demonstrated significantly lower glucose release when astaxanthin increased in the formulation. The results also demonstrated that the inclusion of astaxanthin with wholemeal flour significantly improve the antioxidant properties of the model food.

Chapter 6

Impact of different substrates on short-chain fatty acid production during *in-vitro* bacterial fermentation

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Abstract

Whole-grain cereals, berries and microalgae are major sources of bioactive compounds including dietary fibre. The fibre fraction of previously developed cookies containing different proportions (0, 5, 10 and 15 %) of blackcurrant and astaxanthin-rich microalgae *Haematococcus pluvialis* powder with wholemeal wheat, barley and oat flours were compared. Amongst all the cookies, the 15 % blackcurrant and astaxanthin powder containing cookies had a significantly ($P < 0.05$) higher fibre fraction. Combinations of four bacterial species (*Lactobacillus* and *Bifidobacterium*) were used in a fermentation of the nine different cookies as substrates. Ferment pH significantly ($P < 0.05$) decreased, and optical density (OD₆₀₀) increased, after 24 and 48 h incubation of blackcurrant/astaxanthin incorporated substrates. The concentration of SCFA was slightly higher in the substrate from blackcurrant and astaxanthin cookies compared with wholegrain cereals cookie substrates.

6.1. Introduction

The colonic microbiota of human gastrointestinal tract has been considered as a metabolic organ, which contains approximately 10^{14} commensal bacteria composed of 500 to 1000 different species (number increases with age) (Backhed et al. 2004; Ramakrishna 2007; Huttenhower et al. 2012). The microbial diversity in the colon is due to the metabolic interactions between microbiota (Rabi & Gibson 2002); and their metabolic functions are highly dependent on the type of substrates to be fermented (Bernalier-Donadille 2010; Poeker et al. 2018). The composition of the microbiota and their metabolic end-products are linked to the health and diseases at the host (Sonnenburg &

Bäckhed 2016; Brown & Hazen 2018). The end-products of bacterial fermentation have been identified to reduce the risk of not only cardiovascular diseases which is leading cause of death globally, but also reduce the risk of other diseases, such as type 2 diabetes, colon cancer and inflammatory bowel disease which also regulating satiety helping to prevent obesity (Brennan 2005; Canfora et al. 2015; Aron-Wisnewsky & Clément 2016; O'keefe 2016; Brown & Hazen 2018).

Dietary fibre (DF) has long been appreciated to be a key element of healthy eating, due to its contribution to diverse range of human health and its inverse correlation with leading diet-related chronic diseases including overweight and obesity (Tucker & Thomas 2009). Dietary fibre exerts physiological effects as it passes through the upper gastrointestinal tract and is fermented by colonic microbiota unlike other basic nutrients such as sugar, fat and protein which are digested by the gastrointestinal enzymes (Cummings & Englyst 1987). Potential physiological effects of dietary fibres, such as lowering of blood sugar, regulation of blood cholesterol and prevention of colon cancer, depends on their characteristics, such as bulking, viscosity, water-holding capacity and fermentability (Schneeman 1987). The major metabolites of the colonic bacterial fermentation of DFs are short-chain (C2-C6) fatty acids (SCFAs) such as acetate, propionate, butyrate, lactate and gases such as CO₂, H₂ and CH₄ (Flint et al. 2007; Flint et al. 2012).

These SCFAs have been acknowledged as potent signalling molecules of various cellular mechanisms and are a promising treatment modality of intestinal disorders and chronic diseases (Kim et al. 2017; Nishitsuji et al. 2017). The predominant SCFA is acetate followed by propionate and butyrate and their approximate molar ratio 60:20:20 in the colon, and their production rate and concentration depends on the source and type of substrate, species and bacterial population available in the colon (Topping & Clifton 2001; Hijova & Chmelarova 2007; Fernando et al. 2008). Up to 70 % of the acetate is used by the liver as an energy source and acts as a substrate for cholesterol synthesis, whereas propionate (up to 90 %) acts as a gluconeogenesis inhibitor and inhibit the synthesis of cholesterol in the liver (De Vadder et al. 2014). Butyrate is the major energy source for colonic mucosa and potentially acts on epithelial cells to inhibit cell proliferation and differentiation (epigenetic function) (Gao et al. 2009; De Vadder et al. 2014).

Wholegrain wheat, barley and oat are important sources of basic nutrients and bioactive compounds such as dietary fibre (10-13 %), antioxidants (1-2 %) (Slavin 2004). All these compounds have significant physiological function and potential health benefits. For example, soluble fibre such as β -glucans found in barley and oat delays gastric emptying and glucose diffusion by increasing viscosity and insoluble fibre such as found in whole-grain wheat increase transit time and faecal bulking within the digestive tract (Brennan & Cleary 2005; Fardet 2010). This enables better management of glucose homeostasis (Brennan 2005). Dietary fibres have colonic effect to a host through SCFAs production (O'keefe 2016).

Blackcurrants are one of the richest sources of bioactive compounds among all berries. They contain a high level of a variety of non-nutritive compounds such as phenolic compounds (100-300 mg/100 g) as well as nutritive compounds such as sugars, essential oils, minerals and significant amount of dietary fibre (4.3% including soluble fibre such as pectin) (Sójka et al. 2009; Zheng et al. 2012). These nutritional and non-nutritional bioactive compounds are linked to potential biological functions protecting health and preventing diseases (Ghosh et al. 2007).

Recently, more attention has been given to the seaweed or algal material due to their diverse range of nutrients and health promoting properties. The microalgae *Haematococcus pluvialis* is a widely available single-cell marine algal strain which contains high levels of astaxanthin (4-5 % of dw) (Orosa et al. 2001). The carbohydrate content of *H. pluvialis* microalgae is relatively higher compared to other microalgae (Boussiba & Vonshak 1991). Due to the high proportion of complex carbohydrate microalgae carbohydrate digestibility is low (Bocanegra et al. 2009; Hossain et al. 2017a), enabling better management of energy homeostasis. Oligosaccharides such as xylo-oligosaccharide, fructo-oligosaccharide present in *H. pluvialis* microalgae, which are moderately absorbed in the GIT, act as a substrate (to be fermented) by the colonic microflora (Mussatto & Mancilha 2007).

Taken together, the complex mix of these plant foods compounds exert a synergistic effect on health when consume together (by lowering glucose release and increasing antioxidant capacity); as reported in our previous studies (Hossain et al. 2017a; Hossain et al. 2017b). This part of the thesis

examined the impact of mixed dietary fibres on the production of metabolites in an *in-vitro* bacterial fermentation.

6.2. Materials and Methods

6.2.1. Determination of insoluble and soluble dietary fibre (IDF, SDF)

The cookies were analysed for the IDF and SDF as described in section 3.11.

6.2.2. Total dietary fibre (TDF) isolation

TDF was isolated from the as described in section 3.12.

6.2.3. Bacterial strains

Described in section 3.13.

6.2.4. Bacterial sub-culture and preparation of cell suspensions

Described in section 3.14.

6.2.4. Preparation of PYF growth medium

Described in section 3.15.

6.2.5. *In-vitro* fermentation

Fermentation was carried out as described in section 3.16.

6.2.6. Determination of SCFA

SCFAs was analysed as described in section 3.17.

6.2.7. Statistical analysis

Described in section 3.24.

6.3. Result and discussion

6.3.1. Determination of cookie dietary fibre

The insoluble and soluble dietary fibres affect the technological functionality in different food processing techniques (such as baking, boiling, grinding, frying and extrusion-cooking) and improve physiological outcomes for human health (Sánchez-Alonso et al. 2007). The dietary fibre fractions

(IDF, SDF and TDF) of the cookies are summarized and presented in Table 6.1 and 6.2. The fibre contents were significantly increased ($P < 0.05$) as the blackcurrant and astaxanthin inclusion increased in all flour types. The TDF of control cookies ranged from 8.5 % to 9.5 %, this increased to a maximum of 16 % with the inclusion of 15 % blackcurrant powder in wholemeal wheat flour cookies (Table 1). The IDF is a major DF fraction in all cookies; however SDF was significantly increased in all flour types with the addition of blackcurrant powder and in oat flours with 15 % astaxanthin addition, to a maximum of 3-4 % (DM) (Table 6.2). Analysis of ingredients shows that the blackcurrant and astaxanthin powders contained two to three times more DF compared with the wholemeal flours (Table 6.3), which are in accordance with USDA Database SR28 (Reinisalo et al. 2015).

DF of whole-grain cereals can be categorised into two major groups: arabinoxylan prominent and β -glucans prominent derived from wheat and barley/oats respectively (Frølich et al. 2013). The other DF components of whole-grain cereals are cellulose, fructan and lignin. The different fibre types and their composition have different physico-chemical properties which exert different physiological function. Increased proportions of SDF (such as β -glucan) have been shown to increase viscosity to the gut and decrease the rate of nutrient absorption in the small intestine, bile acid reabsorption and influence metabolic responses such as reduced insulin and reducing blood cholesterol (Queenan et al. 2007). Increasing IDF (such as cellulose) influences gastric emptying, digestive motility and has bulking effects (Kashyap et al. 2013).

Blackcurrant powder showed an IDF/SDF ratio of 67/33, which is in accordance with McCance & Widdowson recommendations (Paul et al. 1980). Consequently, DF derived from blackcurrant may improve the health promoting properties of foods due to the both IDF and SDF ratio compared with the IDF/SDF ratio of cereals. The concentrated DF of blackcurrant consists of cellulose (30 %) and pectin (25 %) (Paul et al. 1980). Principally, fruit sugars are formed by cellulose, and uronic acids from pectin. The high proportion of cellulose and pectin in blackcurrant fibre indicates the diverse range of

physiological properties and disease prevention (such as colorectal cancer, coronary heart disease, type 2 diabetes and obesity), which is incorporated in to the cookies (Anderson et al. 2009).

The TDF content of astaxanthin rich microalgae *Haematococcus pluvialis* is 30 g/100 g (MacArtain et al. 2007), and this is higher than most cereals and fruits fibre content. The composition of microalgae DF varies greatly; they differ physico-chemically from the other plant foods (such as cereals fibre) and thus may exert different physiological effects. The concentrated DF of microalgae constituted such as agar, carrageenan, cellulose and alginates, which have been shown to influence intestinal metabolism due to their mucus composition (gelling effect), pH, SCFAs production and enhance the growth of beneficial bacteria to the gut (O’Sullivan et al. 2010). The concentrated DF fraction of microalgae has been shown to have a diverse range of bioactive properties such as anticancer and hypocholesterolemic activity as well as its therapeutic potentials of IDF and SDF fractions (Pomin & Mourão 2008). The increased of DF in astaxanthin-rich cookies shows that they have potential to exhibit similar bioactive properties.

Table 6.1. Dietary fibre composition of blackcurrant containing cookie

Sample	IDF	SDF	TDF
Wholemeal wheat cookie control	7.56 ± 0.01 ^d	1.88 ± 0.05 ^b	9.44 ± 0.06 ^d
Wholemeal wheat + 5% blackcurrant	11.01 ± 0.08 ^c	1.80 ± 0.22 ^b	12.80 ± 0.13 ^c
Wholemeal wheat + 10% blackcurrant	11.84 ± 0.17 ^b	2.30 ± 0.06 ^{ab}	14.14 ± 0.24 ^b
Wholemeal wheat + 15% blackcurrant	13.83 ± 0.34 ^a	2.85 ± 0.21 ^a	16.69 ± 0.13 ^a
Wholemeal barley cookie control	7.50 ± 0.37 ^c	2.05 ± 0.01 ^b	9.55 ± 0.39 ^c
Wholemeal barley + 5% blackcurrant	9.54 ± 0.20 ^b	2.95 ± 0.12 ^a	12.53 ± 0.46 ^b
Wholemeal barley + 10% blackcurrant	9.80 ± 0.40 ^{ab}	2.98 ± 0.10 ^a	12.74 ± 0.33 ^b
Wholemeal barley + 15% blackcurrant	11.33 ± 0.57 ^a	3.29 ± 0.14 ^a	14.624 ± 0.25 ^a
Wholemeal oat cookie control	6.50 ± 0.13 ^c	2.20 ± 0.05 ^c	8.70 ± 0.19 ^c
Wholemeal oat + 5% blackcurrant	7.02 ± 0.12 ^c	2.32 ± 0.06 ^c	9.34 ± 0.05 ^c
Wholemeal oat + 10% blackcurrant	10.23 ± 0.36 ^b	2.80 ± 0.03 ^b	13.03 ± 0.33 ^b
Wholemeal oat + 15% blackcurrant	11.72 ± 0.29 ^a	3.47 ± 0.19 ^a	15.18 ± 0.49 ^a

Data expressed as g / 100 g sample, DW); *n* = 3, values with different letter in each column are significantly different (*p* < 0.05)

Table 6.2. Dietary fibre composition of astaxanthin containing cookie

Sample	IDF	SDF	TDF
Wholemeal wheat cookie control	7.56 ± 0.017 ^c	1.88 ± 0.05 ^a	9.44 ± 0.06 ^c
Wholemeal wheat + 5% astaxanthin	8.25 ± 0.01 ^c	0.97 ± 0.01 ^c	9.21 ± 0.09 ^c
Wholemeal wheat + 10% astaxanthin	10.67 ± 0.12 ^b	1.38 ± 0.07 ^b	12.06 ± 0.05 ^b
Wholemeal wheat + 15% astaxanthin	12.42 ± 0.39 ^a	1.83 ± 0.03 ^a	14.24 ± 0.43 ^a
Wholemeal barley cookie control	7.49 ± 0.37 ^c	2.06 ± 0.01 ^{ab}	9.55 ± 0.39 ^b
Wholemeal barley + 5% astaxanthin	8.42 ± 0.37 ^{bc}	1.50 ± 0.02 ^b	9.93 ± 0.40 ^b
Wholemeal barley + 10% astaxanthin	8.89 ± 0.13 ^b	1.83 ± 0.18 ^{ab}	10.72 ± 0.04 ^b
Wholemeal barley + 15% astaxanthin	11.67 ± 0.09 ^a	2.34 ± 0.27 ^a	14.01 ± 0.18 ^a
Wholemeal oat cookie control	6.49 ± 0.13 ^c	2.19 ± 0.05 ^b	8.69 ± 0.19 ^c
Wholemeal oat + 5% astaxanthin	6.67 ± 0.16 ^c	2.61 ± 0.01 ^b	9.29 ± 0.15 ^c
Wholemeal oat + 10% astaxanthin	7.67 ± 0.03 ^b	2.69 ± 0.22 ^b	10.35 ± 0.26 ^b
Wholemeal oat + 15% astaxanthin	9.34 ± 0.12 ^a	3.41 ± 0.11 ^a	12.78 ± 0.23 ^a

Data expressed as g / 100 g sample, DW); $n = 3$, values with different letter in each column are significantly different ($p < 0.05$)

Table 6.3. Dietary fibre composition of raw samples of cookie (g / 100 g sample, DW)

Raw Sample	IDF	SDF	TDF
Wholemeal Wheat Flour	13.33 ± 0.05	2.05 ± 0.01	15.39 ± 0.07
Wholemeal Barley Flour	7.57 ± 0.12	3.98 ± 0.01	11.56 ± 0.13
Wholemeal Oat Flour	7.87 ± 0.09	4.16 ± 0.08	12.04 ± 0.17
Blackcurrant Powder	16.83 ± 0.37	8.37 ± 0.57	25.20 ± 0.94
Astaxanthin Powder	25.24 ± 0.11	5.39 ± 0.27	30.63 ± 0.38

6.3.2. Evaluation of bacterial growth

In-vitro study of the combined effect of gut microbiota assists in the understanding the mechanism of different substrates and the effect that their metabolic end-products production have on population dynamics in the intestinal microbiota. The bacterial growth and viability indicate the ability of the microbes to utilise the cookie substrate. The ability of the bacteria to grow on the DF from the cookies and the carbohydrate free incubated media were evaluated by measuring the pH (Table 6.4) and optical density (OD) as shown in Table 6.5. Such measurements have previously been correlated with fermentability (Fernando et al. 2011). The pH value decreased significantly with time, caused by acid production associated with bacterial growth and OD value increased significantly with time throughout the fermentation, suggesting that the cookie fibres are fermented by bacteria.

The change in pH and optical density represents a change in the microbial population and potentially the relationships between different microbial species but not necessarily the total number of microbes. Microbial growth (pH and OD data) of each of the substrates was statistically different ($P < 0.05$) at 0, 6, 24 and 48 h fermentation. Comparatively low pH values were observed on substrate from blackcurrant then astaxanthin and wholemeal cereal containing cookie among three groups but relatively low pH were observed on positive control (glucose). The cookies provide a substrate combination that is thought to promote the growth of *Lactobacillus* species and *Bifidbacterium* species. These microbes are considered to inhibit the growth of potentially harmful microbes (such as *Clostridium difficile*, *E. coli* and *Ruminococcus callidus*) and protective against gastrointestinal diseases (such as ulcerative colitis, inflammatory bowel disease and crohn's disease) to the host (Gotteland et al. 2001).

Table 6.4. Effect of cookie fibre on microbial growth (evaluated by pH)

Sample\Hour	T0	T6	T24	T48
Wholemeal wheat cookie control	6.05 ± 0.04 ^a	5.38 ± 0.00 ^b	4.95 ± 0.03 ^c	4.71 ± 0.02 ^d
Wholemeal wheat+15% Blackcurrant	6.05 ± 0.01 ^a	5.35 ± 0.02 ^b	4.86 ± 0.02 ^c	4.66 ± 0.01 ^d
Wholemeal wheat+15% astaxanthin	6.05 ± 0.01 ^a	5.36 ± 0.03 ^b	4.90 ± 0.01 ^c	4.705 ± .01 ^d
Wholemeal barley cookie control	5.97 ± 0.04 ^a	5.29 ± 0.01 ^b	4.98 ± 0.02 ^c	4.77 ± 0.02 ^d
Wholemeal barley+15% Blackcurrant	5.97 ± 0.01 ^a	5.28 ± 0.01 ^b	4.97 ± 0.01 ^c	4.70 ± 0.03 ^d
Wholemeal barley+15% astaxanthin	5.95 ± 0.02 ^a	5.30 ± 0.01 ^b	4.99 ± 0.00 ^c	4.75 ± 0.04 ^d
Wholemeal oat cookie control	5.96 ± 0.04 ^a	5.28 ± 0.01 ^b	5.00 ± 0.03 ^c	4.75 ± 0.03 ^d
Wholemeal oat+5% Blackcurrant	5.95 ± 0.00 ^a	5.28 ± 0.0 ^b	4.97 ± 0.01 ^c	4.71 ± 0.01 ^d
Wholemeal oat+15% astaxanthin	5.96 ± 0.01 ^a	5.28 ± 0.00 ^b	5.00 ± 0.01 ^c	4.74 ± 0.01 ^d
Positive control	5.96 ± 0.04 ^a	5.27 ± 0.01 ^b	4.91 ± 0.00 ^c	4.68 ± 0.01 ^d
Negative control	7.65 ± 0.01 ^a	7.64 ± 0.01 ^a	7.64 ± 0.01 ^c	7.65 ± 0.01 ^a

Data were compared within different fermentation hours; $n = 3$, values with different letter in each column are significantly different ($p < 0.05$)

Table 6.5. Effect of cookie fibre on microbial growth (evaluated by OD600)

Sample\Hour	T0	T6	T24	T48
Wholemeal wheat cookie control	0.566 ± 0.00 ^d	0.663 ± 0.00 ^c	1.065 ± 0.03 ^b	1.445 ± 0.01 ^a
Wholemeal wheat +15% Blackcurrant	0.563 ± 0.00 ^d	0.663 ± 0.00 ^c	1.156 ± 0.02 ^b	1.569 ± 0.00 ^a
Wholemeal wheat+15% astaxanthin	0.565 ± 0.00 ^c	0.663 ± 0.00 ^c	1.138 ± 0.04 ^b	1.454 ± 0.05 ^a
Wholemeal barley cookie control	0.553 ± 0.01 ^d	0.659 ± 0.00 ^c	1.040 ± 0.01 ^b	1.493 ± 0.01 ^a
Wholemeal barley+15% Blackcurrant	0.549 ± 0.00 ^c	0.659 ± 0.00 ^c	1.079 ± 0.04 ^b	1.538 ± 0.03 ^a
Wholemeal barley+15% astaxanthin	0.550 ± 0.01 ^d	0.655 ± 0.01 ^c	1.067 ± 0.01 ^b	1.525 ± 0.03 ^a
Wholemeal oat cookie control	0.551 ± 0.00 ^d	0.662 ± 0.00 ^c	1.074 ± 0.01 ^b	1.427 ± 0.03 ^a
Wholemeal oat+5% Blackcurrant	0.548 ± 0.00 ^d	0.666 ± 0.00 ^c	1.125 ± 0.02 ^b	1.536 ± 0.01 ^a
Wholemeal oat+15% astaxanthin	0.547 ± 0.00 ^d	0.665 ± 0.01 ^c	1.072 ± 0.00 ^b	1.460 ± 0.01 ^a
Positive control	0.549 ± 0.00 ^d	0.670 ± 0.00 ^c	1.178 ± 0.01 ^b	1.528 ± 0.01 ^a
Negative control	0.003 ± 0.00 ^a	0.001 ± 0.00 ^a	0.004 ± 0.00 ^a	0.005 ± 0.00 ^a

Data were compared within different fermentation hours; $n = 3$, values with different letter in each column are significantly different ($p < 0.05$)

6.3.3. Determination of short-chain fatty acids production

The human gut microbiota utilises dietary residues as substrates for energy and growth (Gibson et al. 1995). SCFAs are the metabolites (> 95 %) produced by the microbiota. The concentration of SCFA increased significantly from 24 h to 48 h for fermentation of all cookie substrates (Table 6.6), other research has found that the higher rate of bacterial growth occurs after 24 h (Fernando et al. 2008). Acetate had the highest concentration among measured SCFAs, followed by butyrate and propionate. The fermentability and rate of SCFAs production of blackcurrant and astaxanthin incorporated cookies substrate demonstrated slightly higher when compared with wholemeal flour cookies substrate during the fermentation. Barley cookie substrate exhibited higher trend of SCFA production among all cookie groups. This could be due to the β -glucans present in barley which is readily fermented than arabinoxylans of wheat. Arabinoxylans is composed of xylose and arabinose subunits and the strong bonding of the structure of whole-grain wheat DF has an effect on the bacterial fermentation (Stevens et al. 1988). The xylose appears to enable it to resist fermentation. Thus wheat DF appears less susceptible to fermentation than barely or oat DF. In the human gut arabinoxylan may be more readily fermented by other microbiota. The differences in SCFA production between the different substrates could be due to the differences in composition such as IDF and SDF ratio and physico-chemical nature (such as solubility) of the substrate affecting

utilisation by the bacteria (Fernando et al. 2018). The pattern of SCFA production from the barley group substrate were acetate>propionate>butyrate which is in agreement with other research (McBurney & Thompson 1990). However, the production of propionate by blackcurrant containing barley group was higher as compared with other (wheat and oat cookie) substrates. The higher production of butyrate by wheat and oat cookie substrates is in agreement with other research (Karppinen et al. 2000). However, both *in-vivo* and *in-vitro* studies have demonstrated that species variation in fermentation, source of substrate and substrate compositions affect in SCFA production found in other research (Cummings & Englyst 1987; McBurney & Thompson 1990; Titgemeyer et al. 1991; Karppinen et al. 2000; Fernando et al. 2008).

Table 6.6. SCFA (mmoles/100 mL) of *in-vitro* bacterial fermentation (combination of four bacterial strains) of cookie fibre (TDF)

Sample\ Hour	Acetate				Propionate				Butyrate			
	0	6	24	48	0	6	24	48	0	6	24	48
WCC	0.721±0.01	1.011±0.12	1.094±0.09	1.657±0.18	0.023	0.023	0.037	0.047	0.03	0.03	0.051	0.055
W15B	0.704±0.03	1.022±0.19	1.175±0.11	1.657±0.22	0.021	0.027	0.044	0.049	0.03	0.032	0.053	0.053
W15A	0.683±0.01	0.968±0.11	1.094±0.08	1.629±0.13	0.016	0.022	0.037	0.044	0.028	0.032	0.052	0.053
BCC	0.779±0.06	1.074±0.09	1.23±0.08	1.74±0.16	0.021	0.022	0.035	0.048	0.024	0.027	0.046	0.048
B15B	0.718±0.02	1.049±0.11	1.204±0.11	1.81±0.19	0.018	0.022	0.041	0.062	0.027	0.027	0.055	0.055
B15A	0.734±0.01	1.029±0.13	1.138±0.15	1.777±0.16	0.017	0.022	0.041	0.054	0.025	0.026	0.055	0.057
OCC	0.773±0.01	1.089±0.11	1.223±0.12	1.89±0.18	0.021	0.02	0.04	0.044	0.024	0.027	0.049	0.054
O15B	0.764±0.08	1.000±0.09	1.171±0.12	1.893±0.13	0.02	0.023	0.04	0.046	0.026	0.027	0.049	0.048
O15A	0.770±0.01	1.077±0.09	1.184±0.11	1.895±0.22	0.021	0.023	0.04	0.05	0.027	0.028	0.05	0.053
CON(+)	0.733±0.04	1.068±0.10	1.2±0.06	1.858±0.21	0.02	0.022	0.037	0.041	0.026	0.028	0.049	0.055
CON (-)	0.139±0.00	0.147±0.00	0.134±0.01	0.14±0.00	0.024	0.02	0.024	0.028	0.021	0.023	0.021	0.022

WCC- wholemeal wheat cookie control, W15B- wholemeal wheat + 15 % blackcurrant, W15A- wholemeal wheat + 15 % astaxanthin, BCC- wholemeal barley cookie control, B15B- wholemeal barley + 15 % blackcurrant, B15A- wholemeal barley + 15 % astaxanthin, OCC- wholemeal oat cookie control, O15B- wholemeal oat + 15 % blackcurrant, O15A- wholemeal oat + 15 % astaxanthin, CON (+) – positive control, CON (-) – negative control

6.4. Conclusion

It is important to understand the composition of dietary sources so that they can be effectively utilised in order to enhance the functionality of foods thus increasing health benefits to the consumer. This study showed the effect of replacement of wholemeal flours (wheat, barley and oat) with either blackcurrant powder or astaxanthin-rich microalgae (*H. pluvialis*) powder. Inclusion of blackcurrant or astaxanthin powder impacted positively on health promoting parameters, including increasing SDF and IDF as well as enhancing the growth of beneficial human gut bacteria and increasing the concentration of SCFA in an *in-vitro* fermentation. Blackcurrant powder and astaxanthin powder not only contribute DF but have high levels of vitamins, minerals and phytonutrients which play a protective role against CHD, type 2 diabetes, obesity and some cancers. The gut microbiota is diverse and is affected by the substrate supplied. The characteristics and combination of microbes are responsible for producing the state of the gut due to their metabolism. However, it has been shown here that providing the microbiota with an appropriate substrate leads to the production of a healthier gut environment.

Chapter 7

Role of fermented metabolites in oxidation reduction and epigenetic regulation

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Abstract

Obesity is a multifactorial disorder for early disability including several dysfunctions such as inflammation and energy metabolism, resulting from complex biological interaction between genetic and environment factors. Bioactive compounds of a diet especially, dietary fibre, can be attributed to the genetic changes, leading to obesity prevention. We investigated the bioactivity of fermented metabolites in cellular antioxidant activity and changes in genomic regulation, in relation to anti-obesity and inflammation. The fermented metabolites of wholemeal wheat cookies fortified with 15 % blackcurrant (W15B) and wholemeal barley incorporated 15 % blackcurrant cookies (B15B) showed greater antioxidant activity in responses cellular antioxidant analysis (CAA) and ORAC determinations, and inhibition of proliferation than the wholemeal wheat cookie control (WCC) and wholemeal barley cookie control (BCC), in HepG2 cell model. Importantly, qRT-PCR exhibited the significantly higher mRNA levels of satiety genes (ten to twelve-fold) and lower levels of inflammatory cytokines (half to two-fold) in W15B and B15B treated HepG2 cells, which could be therapeutic alternative of anti-obesity.

7.1. Introduction

Obesity (body mass index, BMI > 30 kg/m²) is a heterogeneous medical condition with many associated comorbidities, leading to increase morbidity and mortality (Poirier et al. 2006; Heymsfield & Wadden 2017). The epidemic surge of obesity has been associated with a number of major chronic diseases including metabolic disorder, cardiovascular diseases (number one cause one of death worldwide), insulin resistance, type 2 diabetes (7th), neurodegeneration, aging and all form of cancers

(2nd) (Poirier et al. 2006; WHO 2015; DeBoer et al. 2017; Freisling et al. 2017; Sanguinetti et al. 2018). Diet is thought to be one of the most important factors regulating obesity (with excessive energy intake accounting for 20 -60 % of chronic diseases) (Doll 1992; Jensen et al. 2016). Excessive energy stored as triglyceride in adipose tissue increases the risk to adverse health consequences. It is well established from numerous animal model and human studies that complex interactions of gut endocrine systems and nutrient sensing signals physiologically affect appetite control and satiety (Small & Bloom 2004; Mishra et al. 2016). Dietary changes such as high fat and less bioactive food attenuate the coherent physiological systems, impaired energy homeostasis, increases appetite and obesity (Dow et al. 2012; Jensen et al. 2016). Numerous studies have demonstrated that specific diet particularly bioactive diet stimulates gut nutrient sensor to alterations in circulating the hormones. For example gut hormone cholecystokinin (CCK) that is released during the course of bioactive food ingestion, leading to control meal size, potentially circulating satiety signals and prevent obesity (Kim et al. 2018). Upregulation of gastrin-releasing peptide (such as bombesin) delaying gastric emptying time, reduce appetite and implicated in obesity and cancer, have found in obese rats (Mhalhal et al. 2017).

Dietary fibre (DF) has long been appreciated due to their potential inverse relationship with major chronic diseases. DFs are composed of a wide variety of substances, particularly polysaccharides (such as cereals fibre) and polyphenols (such as fruits fibre) (Pérez-Jiménez & Saura-Calixto 2017). Previous studies have been reported that fruit fibre exerts potential health benefits due to their better nutritional quality, particularly biologically active compounds including polyphenols and fibre polysaccharides than those from cereals fibre (Wang et al. 2016; Pérez-Jiménez & Saura-Calixto 2017).

Gut microbiota and metabolites influences the immunity and diseases susceptibility (Wu et al. 2014a). Fermented metabolites of DFs, particularly SCFAs play a central role to the gut health and the body (Hooper et al. 2012). SCFAs affect a numerous physiological process in multiple organs and/or peripheral tissues, produce signal to distant organs, effects on appetite regulation, immunity (suppresses inflammation) and epigenetic modulation (Kaczmarczyk et al. 2012; Byrne et al. 2015).

Moreover, increased amounts of DF in a diet modulate the composition of gut microbiota, colonic epithelial cells growth and protect host from pathogenic infections (Marques et al. 2017). However, the rate, ratio and level of SCFAs production is a complex interplay between type of substrate and diversity of gut microbiota (Peng et al. 2013; Marques et al. 2017). Although the potential role of consumption of high fibre diet in a wide range of health benefits have been widely studied, but the bioactive gut metabolites in cellular biological regulation for weight management is a major unaddressed challenge.

Given the importance of bioactive metabolites in physiological processes, current study was designed to evaluate the cellular antioxidant, anti-proliferation activity and regulation of digestive and inflammation-related genes expression on human cell model, HepG2, which may contributes to the understanding of biological activities of different substrate metabolites and their synergistic effects at cellular level.

7.2. Materials and methods

7.2.1. Chemicals and reagents

Described in section 3.1

7.2.2. Test compounds

This study was used the fermented metabolites as described in section 3.16.

7.2.3. Determination of antioxidant content

Described in section 3.8.

7.2.4. Chemical antioxidant analysis

The oxygen radical absorbance capacity (ORAC) assay as described in section 3.9.2.

7.2.5. Cell culture and culture condition

Described in section 3.18.

7.2.6. Cellular antioxidant activity (CAA) of HepG2 cells for the fermented metabolites

Described in section 3.19.

7.2.7. Cytotoxicity and inhibitory effects on the proliferation

Described in section 3.20.

7.2.8. Quantitative real-time PCR and regulation of gene expression

Described in section 3.21.

7.2.9. Statistical analysis

Described in section 3.24.

7.3. Result and discussion

7.3.1. Antioxidant content and chemical antioxidant analysis

This study revealed the biological significance of fermented metabolites of different substrate as they have higher amount of targeted bioactive molecules. The most abundant bioactive phenolics (expressed as gallic acid equivalents) were observed in W15B and B15B samples Table 7.1. The increased level of antioxidant contents of W15B (2.35 ± 0.10 mg GAE/g) and B15B (2.45 ± 0.03 mg GAE/g) tested samples could be due to the incorporation of blackcurrant, as fruit fibre is a complex mixture of polysaccharides and polyphenols which might be released during the bacterial fermentation (Wawer et al. 2006). Importantly, these substrates are degraded by the colonic microbiota and may exert antioxidant potential, as previous studies have linked microbiome-derived bioactive phenolics to the gut health and colon cancer prevention (Bravo 1998). However, the potential health benefits of bioactive molecules depend on their bioavailability (Rein et al. 2013).

Table 7.1. Total bioactive phenolics and chemical antioxidant activity of fermented metabolites

Sample	TPC value	ORAC value
WCC	1.35 ± 0.02^c	62.78 ± 4.38^d
W15B	2.35 ± 0.10^a	122.20 ± 5.12^b
BCC	1.53 ± 0.01^b	70.62 ± 3.97^c
B15B	2.45 ± 0.03^a	127.90 ± 5.66^b

Data expressed as TPC: MEAN mg GAE/ g sample, DW; $n = 3$; ORAC: MEAN NetAUC Trolox equivalents ($\mu\text{mol TE/g sample, DW}$); $n = 3$ values with different letter in each column are significantly different ($p < 0.05$)

Antioxidant activity is thought to be one of the major bioactive factors, which is positively associated with the phenolic content. The total antioxidant activities of different substrate metabolites were assessed by oxygen radical absorbance capacity using ORAC values, as peroxy radical of ORAC assay produce deleterious effects in the human body. The result in table 7.1 demonstrate that all substrate metabolites exerted peroxy radical scavenging activity *in-vitro*. The most potent metabolites were found from W15B and B15B fraction as they presented higher ORAC value of $122.20 \pm 5.12 \mu\text{M TE/g}$ and $127.90 \pm 5.66 \mu\text{M TE/g}$ compared to the other samples. The significantly higher ORAC value of the metabolites indicated that the tested samples contain higher bioactive phenolics likely possess better protection to detoxify or scavenge those free radicals. Importantly, besides the high concentrations of particular antioxidants, the synergism between components and redox properties might be the important factors assist in physiological defences (Phan et al. 2017). However, the chemical antioxidant activities could be dissimilar in complex biological mechanism, as antioxidant compounds are working in a complex physiological condition at cellular level.

7.3.2. Cellular antioxidant activity

Cellular homeostasis and modulation of oxidative stress is a fundamental aspect of potential health protection strategy. Biochemical analysis in cell systems, fermented metabolites were potentially regulated the reactive oxidant molecules, due to their bioactive compounds ability to reduce oxidation of DCFH in intracellular biological system. In the reaction with DCFH-DA, ABAP induced oxidation to fluorescent DCF, which represents the rate of oxidation. The reactions with different fermented metabolites induced changes in the excitation and emission fluorescence of the probe, which confirmed the cellular uptake of tested compounds.

Table 7.2. Cellular antioxidant activity (CAA) of fermented metabolites

Sample	No PBS wash	PBS wash
WCC	378.18 ± 9.63^d	138.59 ± 8.41^d
W15B	711.29 ± 21.34^b	255.71 ± 8.15^b
BCC	404.31 ± 9.14^c	144.59 ± 6.04^c
B15B	796.94 ± 26.60^a	298.61 ± 11.73^a

Data expressed as $\mu\text{mol QE/ 100 g sample, DW}$; $n = 3$, values with different letter in each column are significantly different ($p < 0.05$)

Importantly, fermented metabolites of W15B and B15B exhibited significantly ($p < 0.05$) higher (about 2 times) antioxidant activities, by inhibiting the oxidation than WCC and BCC, in HepG2 cell model; data presented in Table 7.2. The significant CAA differences between the tested samples could be due to the bioavailability of specific potent molecules and/or synergistic interaction of the available compounds at the cellular biological system (Xing et al. 2015). However, WCC and BCC showed a dose-dependent antioxidant activity. As reported previously, fruit fibre fractions are complex mixture of polyphenols and polysaccharides (Pérez-Jiménez & Saura-Calixto 2017), thus, it can be postulated that the higher CAA value of W15B and B15B due to the blackcurrant fraction making a potent intracellular antioxidant. Bioactive phenolics such as catechine, quercetin, delphinidin and kaemferol have been identified as potent antioxidant of blackcurrant, which could be responsible for the higher cellular antioxidant property (Wawer et al. 2006; Nour et al. 2013). These finding revealed the greater protection offered by bacterial metabolites of blackcurrant incorporated fibre fractions against oxidative stress, which could prevent mutation and genetic damage.

7.3.3. Inhibition of proliferation and cytotoxicity

Abnormal cellular growth is the primary pathophysiology of many carcinogenesis (Hamza et al. 2018). The effects of bacterial fermented metabolites on the cellular proliferation were evaluated in human hepatocellular liver carcinoma, HepG2 cell model. Anti-proliferative effects of tested samples were expressed as median effective concentration (EC_{50}), where lower EC_{50} values indicate higher inhibition of proliferation capacity; data are presented in Table 7.3. Fermented metabolites showed significantly higher growth inhibitory effects compared to the control group (Figure 7.1). Importantly, cells treated with W15B and B15B were exhibited significantly ($p < 0.05$) reduced cellular growth with different doses of metabolites, which is in agreement with previous researches (Bishayee et al. 2011; Diaconeasa et al. 2015). The higher inhibitory effects of cell proliferation indicate that metabolites contain such bioactive compounds which have ability to block phase in biological mechanism of cell division and down regulate the cellular growth. Nevertheless, cells treated with WCC and BCC metabolites induced inhibitory effects at higher dose, as the results were shown higher median effective concentration (EC_{50}) means lower effects. However, a similar trend of the potential anti-

proliferative effects of bacterial metabolites and its bioactive molecules in cancerous cell lines was evident and reported previously (Zitvogel et al. 2017).

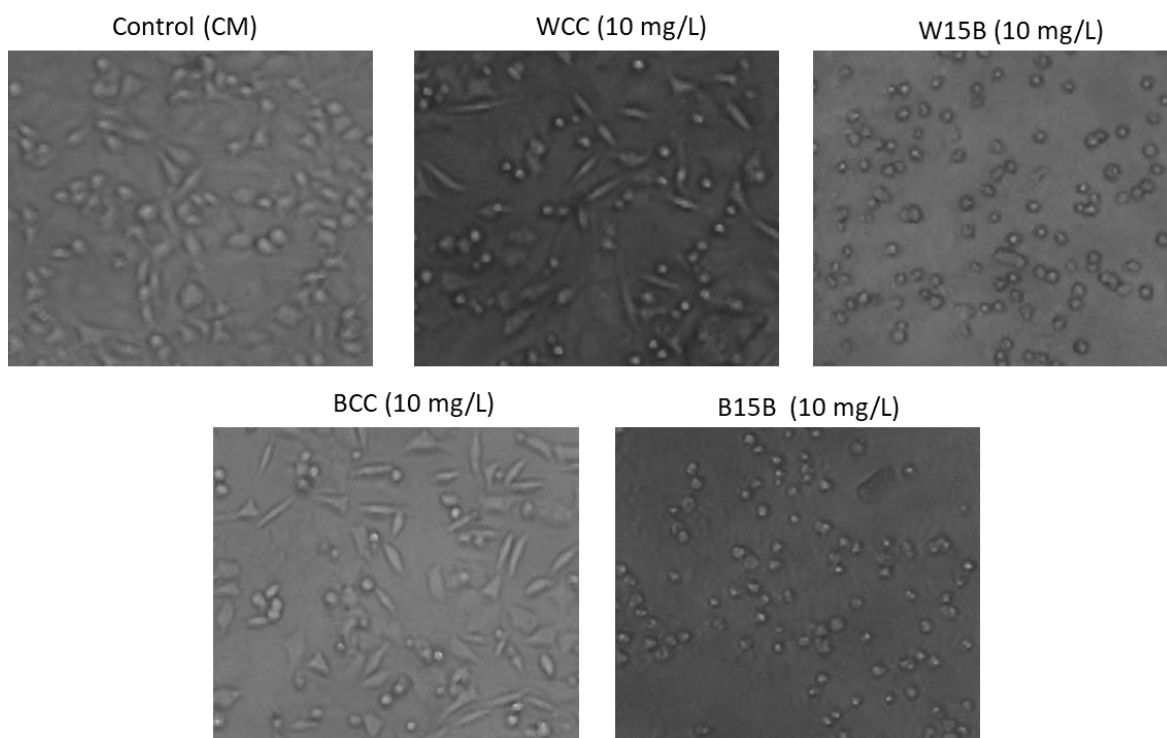


Figure 7.1. Anti-proliferative activity on HepG2 cell line (under microscope observation). Cells treated with SCFAs for 72 h

Table 7.3. Anti-proliferative activity and cytotoxicity of antioxidant-DF fraction on HepG2 cell line

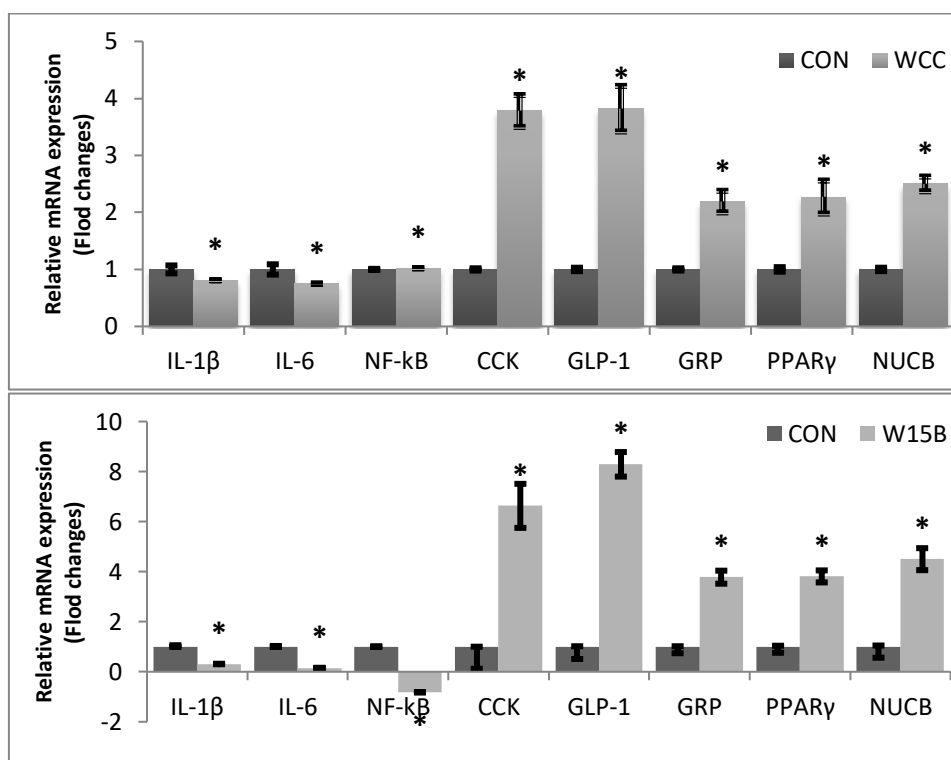
Sample	Anti-proliferation	Cytotoxicity
	EC ₅₀ (mg/mL)	CC ₅₀ (mg/mL)
WCC	16.89 ± 1.32 ^a	>150
W15B	4.17 ± 0.22 ^c	>150
BCC	14.03 ± 1.28 ^b	>150
B15B	3.05 ± 0.17 ^d	>150

Data expressed as Anti-proliferation: median effective concentration (EC₅₀), mg/ mL sample, DW; Cytotoxicity: cytotoxic concentration (CC₅₀), mg/ mL sample, DW; *n* = 3, values with different letter in each column are significantly different (*p* < 0.05)

The cytotoxicity of all the tested samples was evaluated under microscopic observation and optical density analysis using methylene blue. The results showed that the fermented metabolites did not induce any toxic effects with different doses of metabolites on HepG2 cells, compared with control group.

7.3.4. Regulation of gut and inflammatory genes expression

Genetic instability is the key feature of tumour progression, activation of carcinogenesis and immune-mediated diseases. IL-1 β , IL-6 and NF-kB, are widely studied inflammatory markers and increase upon elevated level of ROS production (Jurk et al. 2014). In our study, metabolite treatments in HepG2 cells significantly attenuated the oxidative stress markers. In particular, cells treated with W15B and B15B metabolites significantly suppressed mRNA levels of IL-1 β and IL-6 (about 50 % lower). Additionally, W15B and B15B metabolites were able to significantly down-regulated (about 50 to 90 % lower) the expression of inflammatory transcription factor NF-kB compared to WCC and BCC, (Figure 7.2). The results indicated that the microbe-derived metabolites contain signalling molecules, which have an immunomodulatory effects and coordinated inflammatory cytokine regulation. Importantly, fruit fibre fractions consist of polysaccharides and polyphenols, which could be released during fermentation, and may exert biological effects in systemic inflammation (Roopchand et al. 2015). The exposure of gut microbiota-derived SCFAs modulate systemic effects including immunomodulation, have been reported previously (Delzenne et al. 2011; González-Sarrías et al. 2017). Moreover, down-regulation of inflammatory cytokines by W15B and B15B might be due to the suppressing of NF-kB signalling (Gupta et al. 2014).



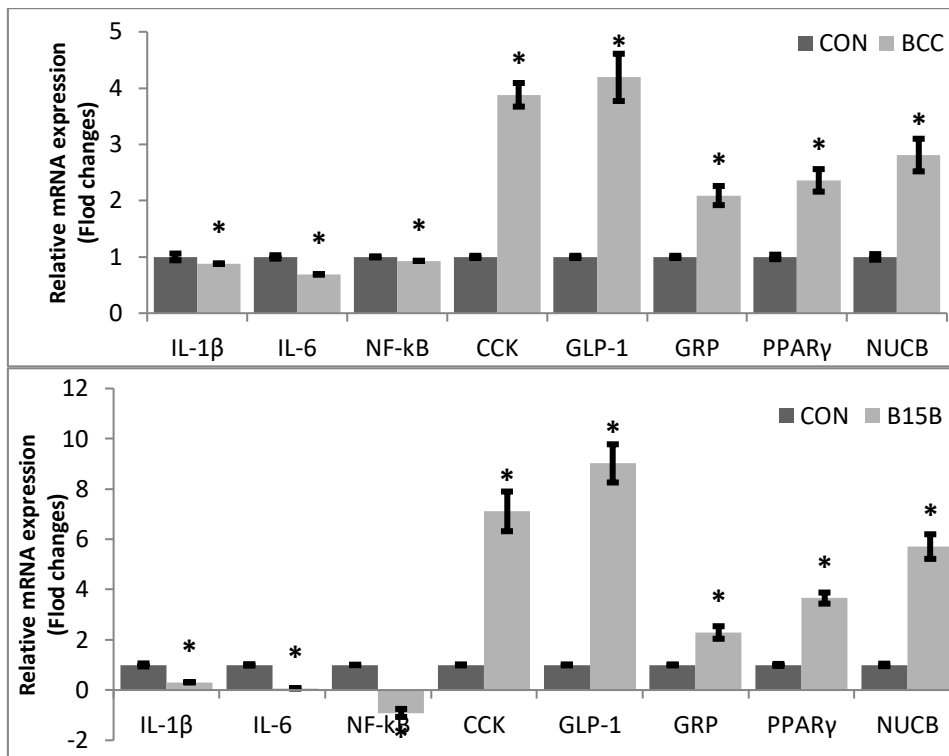


Figure 7.2. Relative mRNA expression (Fold change) of IL-1 β , IL-6, NF-kB, CCK, GLP-1, GRP, PPAR γ , and NUCB after treated with WCC, W15B, BCC and B15B bacterial metabolites (10 mg/ L) for 12 hours. $n = 3$, within each figure, those marked with (*) are significantly different at $p < 0.05$

Cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), gastrin-releasing peptide (GRP), nucleobindin-2 (NUCB2)/nesfatin-1 and peroxisome proliferator-activated receptor (PPAR) are widely studied anorexigenic gene products, key regulator of appetite control, energy homeostasis and satiety. The up-regulation of these anorexigenic gene expression involved in appetite and controlling food intake, have been studied numerous previous studies (Delzenne et al. 2010; Rask-Andersen et al. 2010). Results demonstrated that the metabolites significantly affected the mRNA expression level in HepG2 cell model (Figure 7.2). In particular, the level of CCK, GLP-1 and NUCB2/nesfatin-1 were significantly higher (four to nine fold higher) in the W15B and B15B metabolite treatment compared to the control samples. Nonetheless, WCC and BCC metabolite treatment also enhance the mRNA expression level of GLP-1 and NUCB2/nesfatin-1 (two to four fold higher) significantly ($P < 0.05$), compared to the control. PPAR γ and bombesin-related GRP genes expression were also significantly up-regulated by the all metabolite treatment. These effects could be accounted for the SCFAs-mediated stimulation, which have a biological role in the gut hormone release, have been well documented (Kimura et al. 2013; Psichas et al. 2015b).

Generally, signalling molecules particularly propionate and butyrate have been demonstrated to have higher affinity to the particular receptor such as FFAR, triggering gut-derived hormone including CCK, playing a role in gastrointestinal physiology, facilitated digestion and energy homeostasis (Akiba et al. 2015). Microbiota-derived metabolite butyrate is a potential activator of PPAR γ , enhances gut health, immunity and suppress adipogenesis (Alex et al. 2013). Several studies have identified NUCB2/nesfatin-1 anorexigenic hormone, play a potential role in glucose metabolism, energy homeostasis and decrease body weight (Oh et al. 2006). Higher acetate levels are also positively associated with GLP-1 regulation, potent gut-derived hormone improved glucose tolerance and energy homeostasis (Aoki et al. 2017). In humans, up-regulation of GRP has been reported triggering the release of other gut-derived hormones including GLP-1 and CCK (Psichas et al. 2015a). Recent evidence demonstrates that elevated level of SCFAs, particularly butyrate enhanced the level of GLP-1 production, which improved the appetite-suppressing satiety and influence the insulin production from intestinal L-cells in rats and human (Yadav et al. 2013), which were accomplished by our present study.

7.4. Conclusion

Taken together, substrate dependent metabolic end-products of gut bacteria significantly manipulated the biological activity in cell model. Our results suggest that the improved bioactivities were mediated by blackcurrant additives of the substrate and/or synergistic effects with wholemeal cereals. However, blackcurrant substrate exerted a powerful alteration between the circulation of metabolites, immunological stimulation, inhibition of cancer cell proliferation and two distinct epigenetic regulations- improved appetite-controlling satiety and suppressed inflammatory cytokine in cell line. Therefore, microbiota-derived metabolites are potential nutritional target in the anti-obesity and related disorders. However, further study of these findings, especially in an animal model remains to be determined.

Chapter 8

Blackcurrant incorporated cookie digesta shows chemical and cellular biological activity against human liver cancer cell line, HepG2

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Abstract

The improved understanding of the underlying mechanisms of oxidative damage and/or chronic diseases is of high priority in dietary research. Although the chemical extraction of biofunctional molecules from different fruits and cereals have been studied extensively, the impact of food processing and digestion on bioactivity has not been studied systematically. The aim of this study was to investigate the biofunctional potential of blackcurrant powder incorporated into wholemeal wheat and barley cookies after simulated *in-vitro* digestion. The incorporation of blackcurrant significantly ($p < 0.05$) increased the total phenolic content (about 60 %) and significantly improved oxygen radical absorbance capacity (about 25 %) of cookie digesta in comparison to the digesta components of cookies prepared without blackcurrant addition. Additionally cellular antioxidant and anti-proliferative activity (lowest EC_{50} value 1.02 mg/mL) on human liver cancer cell model, HepG2 was significantly enhanced. Bioactive metabolites of blackcurrant incorporated cookies significantly suppressed the inflammatory cytokine genes IL-1 β (about 3-fold), IL-6 (about 0.5-fold) and NF-kB (about 2-fold) and upregulated satiety gene NUCB-2/Nesfatin-1 (about 5-fold) compared with wholemeal wheat and barley control cookies. The exerted synergistic effects of this study suggest that there may be a new and effective option to prevent and control chronic diseases in human.

8.1. Introduction

Oxidative stress induced by free radicals is a physiological mechanism that has long been recognised as common denominator of numerous life-threatening diseases such as cancer. Reactive oxygen species (ROS) levels regulate the oxidative stress, which causes the degradation of cellular integrity and tissue functions (Holmstrom & Finkel 2014; Poprac et al. 2017). Dysregulation of normal cellular respiration promotes the release of the highly diffusible ROS free radicals such as super oxide anion (O_2^-), hydroxyl radical (OH) and hydrogen peroxide (H_2O_2), mediating oxidative injury of aging (Vitale et al. 2013), carcinogenesis (Ziech et al. 2011), atherosclerosis (Wang et al. 2017b), metabolic syndrome (Banuls et al. 2017), diabetes (Hasnain et al. 2014) and chronic neuronal disorder such as stroke, Parkinson's (PD) and Alzheimer's disease (AD) (Jenner et al. 1992; Ke & Gibson 2004). The uncontrolled formation of ROS and their deleterious effects (redox reactions) can be neutralized by substances named "antioxidants", which act as enzymatic cofactors and inhibit autoxidation (Drummond et al. 2017).

Berries are rich in bioactive compounds including phenolics, flavonoids, anthocyanins and vitamins (Skrovankova et al. 2015). Meta-analysis studies have shown that the consumption of berries exerts a wide range of biological activities in lowering the risk of chronic diseases like cancer, diabetes, aging and neurodegeneration (Li et al. 2014; Huang et al. 2016). Blackcurrants are one of the richest sources of bioactive compounds compared with other berries, that contain a number of polyphenols, including phenolic acids, flavonoids, flavonols, anthocyanin, proanthocyanidins, vitamins and minerals (Paunovic et al. 2017). Recently, much research has been focussed on blackcurrants due to their richness in bioactive compounds, such that they are considered as a functional food due to their diverse health benefits. No previous studies have demonstrated that blackcurrant exerts a neuroprotective effects against oxidative stress induced neural inflammation in human cell lines.

Cereal grains, including wheat and barley, are an excellent source of basic nutrients mainly carbohydrate and protein. Cereal grains also contain high amounts of bioactive compounds such as fibre, vitamins, minerals and phenolic compounds; however, these functional ingredients are often removed or depleted during grain milling and refining (Slavin et al. 2000). There is a suggestion from

epidemiological studies that the consumption of whole grains might reduce the risk of mortality including developing the type 2 diabetes, cardiovascular diseases, and total and specific cancers (Venn & Mann 2004; Flight & Clifton 2006; Ananthakrishnan et al. 2013), but no particular studies have been conducted to identify the biological activity of whole grains on cell model after digestion. Therefore, the objective of this study was to investigate the synergistic effects of bioactive compound rich cookies on the proliferation of human cancer cells and their cellular antioxidant activity.

8.2. Materials and methods

8.2.1. Chemicals and reagents

Described in section 3.1.

8.2.2. Sample preparation

Previously developed cookies were milled before *in-vitro* digestion as described in section 3.3.

8.2.3. *In-vitro* digestion

Described in section 3.7.2.

8.2.4. Determination of total phenolic content

The total phenolic contents (TPC) of the digested samples were determined as described in section 3.8.

8.2.4. Determination of phenolic acids composition by RP-HPLC

The phenolic acid composition of digested samples were identified and quantified as described in section 3.10.

8.2.5. Measurement of total antioxidant capacity

Oxygen radical absorbance capacity (ORAC) assay was performed as described in section 3.9.2.

8.2.6. Cell culture and culture condition

Described in section 3.18.

8.2.7. Cellular antioxidant activity (CAA) of digesta

Described in section 3.19.

8.2.8. Determination of cytotoxicity and anti-proliferative activity

The cytotoxicity and anti-proliferation activity of digested samples was investigated as described in section 3.20.

8.2.9. Quantitative real-time PCR and regulation of gene expression

Described in section 3.21.

8.2.10. Statistical analysis

Described in section 3.24.

8.3. Result and discussion

8.3.1. Gallic acid equivalent total Phenolic content, TPC

This study investigated the effect of digestion on TPC release from the experimental cookies using a simulated gastrointestinal digestion. The results from different digestion phases during the simulated gastrointestinal digestion system (oral, gastric, intestinal and colon) were compared and are presented in Table 8.1. The TPC value of bioactive cookies was significantly increased ($p < 0.05$) compared at the end of gastrointestinal digestion. The highest TPC release from food matrix was observed after completion of intestinal phase in WCC and BCC. This could be due to polyphenols that are bound to proteins in the wholemeal flour being released at the time of protein and cell wall digestion (Chandrasekara & Shahidi 2012). Proteins in wholemeal flour are digested at different rates from upper to lower gastrointestinal tract and the pH environment of gastrointestinal tract could manipulate the phenolics release (McGhie & Walton 2007). Results from the simulated gastrointestinal digestion study showed a three-fold higher TPC than the organic solvent extraction (methanolic extraction, 70 % v/v). This implies a slow-release of the bound phenolics that are present in the cell wall materials of the wholemeal flour, under different physiological conditions.

The mean TPC value (free and bound phenolics) of the combination of blackcurrant powder with wholemeal flour containing W15B and B15B samples were about two-folds higher than the

wholemeal flour control cookies. The TPC of cookies incorporating blackcurrant significantly increased in the gastric phase and then declined during intestinal and colon digestion. This could be due to the higher flavonoid content of blackcurrant powder, which may be released during upper gastrointestinal digestion and may exert a potential health benefit, such as cytoprotection from the oxidative stress in the GIT (Flores et al. 2014). The lower pH condition of the gastric phase may also enhance the rapid cleavage and release of anthocyanins content of blackcurrant. A significant decrease in TPC concentration was observed after the pancreatic bile salt digestion (mimic of intestinal digestion) in mild alkaline environment. It is noteworthy that the total phenolics were significantly affected (about 15 %) by the change in pH, suggesting the phenolics stability at the pH change, which is in agreement with previous studies (Bermúdez-Soto et al. 2007; Flores et al. 2014). Isomerization and interaction with other food components may also attenuate the stability of TPC concentration during in digestion (Farah et al. 2005). Additionally, bioactive polyphenols act as effective inhibitors of digestive enzymes, carbohydrate and protein binding affinity, for example, complex interaction of α -amylase and proanthocyanidins, free amino acids of protein-phenolics and starch-phenolics interaction (Hossain et al. 2017b). Some of the phenolics may degrade and could be oxidised into other chemicals of the assay (Bermudez-Soto et al. 2007). Light and O₂ also are the two most important factors attenuating the TPC concentration during *in-vitro* digestion (Farah et al. 2005). However, the higher TPC concentration of the bioactive cookies release at different phase of digestion may exert potential in local and systemic health benefits.

Table 8.1. Total phenolic content of cookie digesta

Phase	WCC	W15B	BCC	B15B
Oral	44.37 ± 5.37 ^d	205.23 ± 9.00 ^d	41.04 ± 0.30 ^d	254.25 ± 3.08 ^d
Gastric	141.01 ± 4.79 ^c	375.81 ± 2.92 ^a	112.02 ± 3.32 ^c	376.55 ± 4.24 ^c
Intestine	211.28 ± 1.96 ^a	354.51 ± 3.89 ^b	183.50 ± 2.48 ^a	360.86 ± 4.16 ^a
Colon	193.7 ± 25.00 ^b	310.61 ± 6.97 ^c	162.32 ± 2.23 ^b	311.63 ± 3.64 ^b

Data expressed as MEAN mg GAE/100 g sample, $n = 3$; values with different letter in each column are significantly different ($p < 0.05$)

WCC- wholemeal wheat cookie control, W15B- wholemeal wheat + 15 % blackcurrant powder, BCC- wholemeal barley cookie control, B15B- wholemeal barley + 15 % blackcurrant powder

8.3.2. Total antioxidant activity

The antioxidant activity of bioactive compounds of cookie digesta was determined by oxygen radical absorbance capacity (ORAC) assay (Table 8.2). Blackcurrant with wholemeal wheat and barley flour contained higher levels of phenolics and showed significantly ($P < 0.05$) higher ORAC values. It is noteworthy that the presence of blackcurrant in the cookie resulted in higher total phenolics in the gastric phase, while higher total antioxidant activity of the same samples was exhibited in the intestinal and colon phases. This could be due to the compositional changes of phenolic acid released during the individual digestion phase, although further work is required to evaluate this relationship. Additionally, not all phenolic compounds have greater antioxidant and/or biological properties. For example, the anthocyanins of the blackcurrant might have higher antioxidant activity ability and this may lead to the significant differences observed (Skrovankova et al. 2015). The potential radical scavenging ability strongly increased (about 10 %) by the simulated digestion of wholemeal flour cookie compared with chemical extraction of the previous study (Hossain et al. 2017b). This could be due to the fraction of free/bound phenolics ratio of the samples and their release during digestion (Adom & Liu 2002). However, the mean ORAC value was significantly increased when blackcurrants were incorporated to the wholemeal flour cookie after simulated digestion, which have about 20 % higher peroxy radical neutralizing capacity than the control cookies. Furthermore, the enzymatic hydrolysis of cookie components such as carbohydrate and protein digestion may enhance the release of phenolics that are attributed to potential radical scavenging activity. Thus, the blackcurrant material has greater antioxidant capacity and its incorporation into food could be appropriate protection against diseases associated oxidative stress.

Table 8.2. Extracellular antioxidant activities (ORAC values) of cookie digesta

Digestion phase	WCC	W15B	BCC	B15B
Oral	15.34 ± 3.93 ^d	29.92 ± 4.24 ^d	9.63 ± 2.9 ^d	36.29 ± 10.02 ^d
Gastric	53.76 ± 8.71 ^c	64.74 ± 9.38 ^c	40.12 ± 7.86 ^c	58.65 ± 8.16 ^c
Intestine	89.75 ± 2.56 ^a	114.78 ± 10.60 ^a	89.33 ± 3.54 ^a	110.41 ± 11.23 ^a
Colon	86.51 ± 6.57 ^b	112.4 ± 5.49 ^b	81.28 ± 10.84 ^b	109.10 ± 9.28 ^b

Data expressed as MEAN NetAUC Trolox equivalents ($\mu\text{mol TE/g sample, DW}$); $n = 3$, values with different letter in each column are significantly different ($p < 0.05$)

8.3.3. Quantification of individual phenolic compounds

HPLC analysis was performed to evaluate the individual phenolic acids of simulated gastrointestinal digesta of cookies. The relative distribution of identifiable phenolic acids (PAs) for different sample/digestion phase is listed in Table 8.3. The different chromatographic profiles were observed at the end of different digestion phases of incorporation of blackcurrant in wholemeal flour cookies. Nine common peaks, which had acceptable heights and were clearly separated, were calculated with relative areas of the standard. The number of peaks was generally consistent in same group of samples, but the areas of the common peaks were varied greatly at different phases of the digesta, which indicated the concentration variation of PAs. The mean value of highest total PAs content were observed in B15B (263 mg/100 g DW) and W15B (258 mg/ 100 g DW) digested samples (containing 15 % blackcurrant powder). Among all the identifiable PAs fraction of the wholemeal flour cookie digesta, ferulic acid was the most dominant fraction, ranging from 16.49 to 48.16 mg/100 DW, followed by p-coumaric, p-hydroxybenzoic, vanillic and syringic acid respectively. Sinapic acids were detected only in WCC digesta, while chlorogenic acids were detected in BCC digesta. Quercetin-3-rutinoside was the major phenolic acids in W15B and B15B and ranged from 35.77 to 89.66 mg/100 g DW, followed by quercetin derivatives and kaemferol-3-glucoside, confirming that the incorporation of blackcurrant powder strongly contributed to the total PA in the cookie. The individual PAs quantified in the present study were similar to other previous studies have been reported (Slavin et al. 2000; Vagiri et al. 2013; Shamloo et al. 2017). Oral phase digesta were lower PAs content, this is due to the very short residence time (Zhu et al. 2016). Most of the PAs were exhibited in concentrated form in the simulated lower gastrointestinal digestion phase, indicating the PAs slow release as carbohydrate and protein digestion progressed. A slight loss of PAs was identified in the colon phase, which could be due to transformation of pH to an alkaline environment and unstable properties of PAs (Bermudez-Soto et al. 2007). However, the level of PAs markedly increased with the addition of blackcurrant powder to the cookies, which may have significant antioxidant properties in local and systemic health effects and protect against all forms of cancer including digestive cancer.

Table 8.3. HPLC analysis of phenolic acids composition in cookie digesta

Sample	Phase	p-HA	VA	SA	CA	p-CA	FA	Q-3-R	Q	K-3-G	Total PAs
WCC	Oral	5.39 ± 0.1	4.67 ± 0.5	9.27 ± 0.1	nd	1.7 ± 0.0	21.77 ± 1.3	nd	nd	nd	42.8 ± 2.0
WCC	Gastric	8.46 ± 0.8	17.12 ± 1.1	14.61 ± 0.6	6.75 ± 0.1	7.83 ± 0.8	46.92 ± 2.3	nd	nd	nd	101.69 ± 5.7
WCC	Intestine	16.89 ± 0.8	19.13 ± 2.7	17.42 ± 1.1	5.60 ± 0.1	19.70 ± 0.7	57.51 ± 2.8	nd	nd	nd	136.25 ± 8.2
WCC	Colon	19.82 ± 0.4	19.54 ± 1.3	17.80 ± 0.7	4.72 ± 0.2	16.05 ± 1.3	48.16 ± 1.6	nd	nd	nd	126.09 ± 5.5
W15B	Oral	3.82 ± 0.2	9.14 ± 0.3	3.01 ± 0.1	nd	12.89 ± 0.6	16.17 ± 0.5	66.50 ± 2.1	9.28 ± 0.5	6.21 ± 0.0	127.02 ± 4.3
W15B	Gastric	15.57 ± 1.7	13.96 ± 0.2	14.75 ± 1.1	4.13 ± 0.0	29.00 ± 1.1	40.56 ± 1.4	89.66 ± 2.7	36.22 ± 0.7	25.46 ± 0.8	269.31 ± 9.7
W15B	Intestine	18.45 ± 1.6	13.01 ± 0.1	16.58 ± 0.8	5.14 ± 0.0	31.58 ± 1.6	44.69 ± 1.8	83.00 ± 1.8	27.15 ± 1.6	24.84 ± 0.8	264.44 ± 10.1
W15B	Colon	12.95 ± 0.7	19.73 ± 0.3	17.50 ± 0.1	9.64 ± 0.1	27.40 ± 0.9	43.07 ± 1.5	73.10 ± 1.9	28.79 ± 1.3	26.02 ± 1.8	258.2 ± 8.6
BCC	Oral	8.35 ± 0.8	6.96 ± 0.9	nd	15.87 ± 0.6	2.96 ± 0.0	16.49 ± 0.6	nd	nd	nd	50.63 ± 2.9
BCC	Gastric	12.66 ± 0.8	16.06 ± 0.8	10.71 ± 0.0	19.36 ± 0.8	11.09 ± 0.9	29.32 ± 1.5	nd	nd	nd	99.2 ± 4.8
BCC	Intestine	12.78 ± 0.1	15.69 ± 0.1	6.21 ± 0.0	23.49 ± 0.2	19.23 ± 1.5	27.55 ± 0.9	nd	nd	nd	104.95 ± 2.8
BCC	Colon	10.56 ± 0.2	20.70 ± 0.8	4.59 ± 0.1	16.19 ± 0.4	12.94 ± 0.9	28.12 ± 0.7	nd	nd	nd	147.1 ± 3.1
B15B	Oral	17.16 ± 0.2	8.29 ± 0.0	nd	nd	nd	17.16 ± 0.7	35.77 ± 0.6	9.29 ± 0.1	6.05 ± 0.0	93.72 ± 1.6
B15B	Gastric	27.65 ± 1.3	18.80 ± 0.6	10.71 ± 0.0	2.66 ± 0.1	5.59 ± 0.3	54.49 ± 1.6	85.47 ± 2.2	48.81 ± 1.3	10.89 ± 0.2	265.07 ± 7.6
B15B	Intestine	28.74 ± 1.2	12.95 ± 0.2	4.84 ± 0.1	11.27 ± 0.3	18.37 ± 0.1	42.89 ± 1.9	79.50 ± 1.8	37.17 ± 1.5	24.20 ± 0.2	259.93 ± 7.3
B15B	Colon	29.10 ± 1.7	11.84 ± 0.1	3.21 ± 0.0	17.79 ± 0.1	19.03 ± 1.1	45.47 ± 1.6	75.53 ± 2.6	33.68 ± 0.6	27.51 ± 0.6	263.16 ± 8.4

Data expressed as mg phenolic acid (PA) /100 g sample, DW; $n = 3$, values with different letter in each column are significantly different ($p < 0.05$)

Compounds are presented as: p-HA: p-hydroxybenzoic acid; VA: Vanilic acid; SA: syringic acid; CA: caffeic acid, p-CA: p-coumaric acid, FA: ferulic acid; Q-3-R: quercetin-3-rutinoside; Q: quercetin; K-3-G: kaemferol-3-glucoside

8.3.4. Cytotoxic and anti-proliferative effect against HepG2 cell model

The *in-vitro* anti-proliferative activity and cytotoxicity of cookie digesta were evaluated in a dose dependent manner on the human liver cancer cells, HepG2. The cytotoxic effects were tested after completion of each digestion phase of all samples, by human liver cancer cells, HepG2. The CC_{50} value represents the cytotoxicity of the samples, which was observed to change in a dose-dependent manner. The cytotoxic concentration (CC_{50}) value reached up to 200 mg/mL in the oral and gastric phases and up to 120 mg/mL in the intestinal and colon phase (Table 8.4). The minimal stimulatory effects of lower gastrointestinal digesta could be due to the enzymes (pancreatic bile salt digestion) used in the simulated digestion and/or the biofunctional compounds released in the system (Zhu et al. 2015; Martins et al. 2016).

The concentration with greater activity, with no cytotoxicity, are of interest to potential cellular investigation. Anti-proliferation activities of the tested samples were investigated with the concentration having no cytotoxic effects on HepG2 cells. Results of anti-proliferative effects are presented as effective dose (EC_{50}), where lower EC_{50} value indicates higher inhibition of proliferation (Figure 8.1 and Table 8.4). The digested metabolites from the blackcurrant incorporated samples, W15B and B15B, inhibited cancer cells proliferation at the last phase of the digestion. The EC_{50} values were significantly different after each digestion phase and the lowest EC_{50} value was observed at the colon phase, followed by intestinal, gastric and oral phase. The significantly lowest EC_{50} value of each sample was W15B (1.02 mg/mL), B15B (1.97 mg/mL), WCC (58.92 mg/mL) and BCC (61.92 mg/mL) respectively, this reflects (negative correlation) the cellular antioxidant activity (CAA value) in each digestion phase. Complex cellular antioxidant capacity and individual bioactive compounds present in the digesta could have the potential anti-proliferative effects in human cancer cells (Iwata et al. 2017). In addition, the anti-proliferative effects of the cookie increased might be due the improved cellular uptake by digestion (Guo et al. 2017).

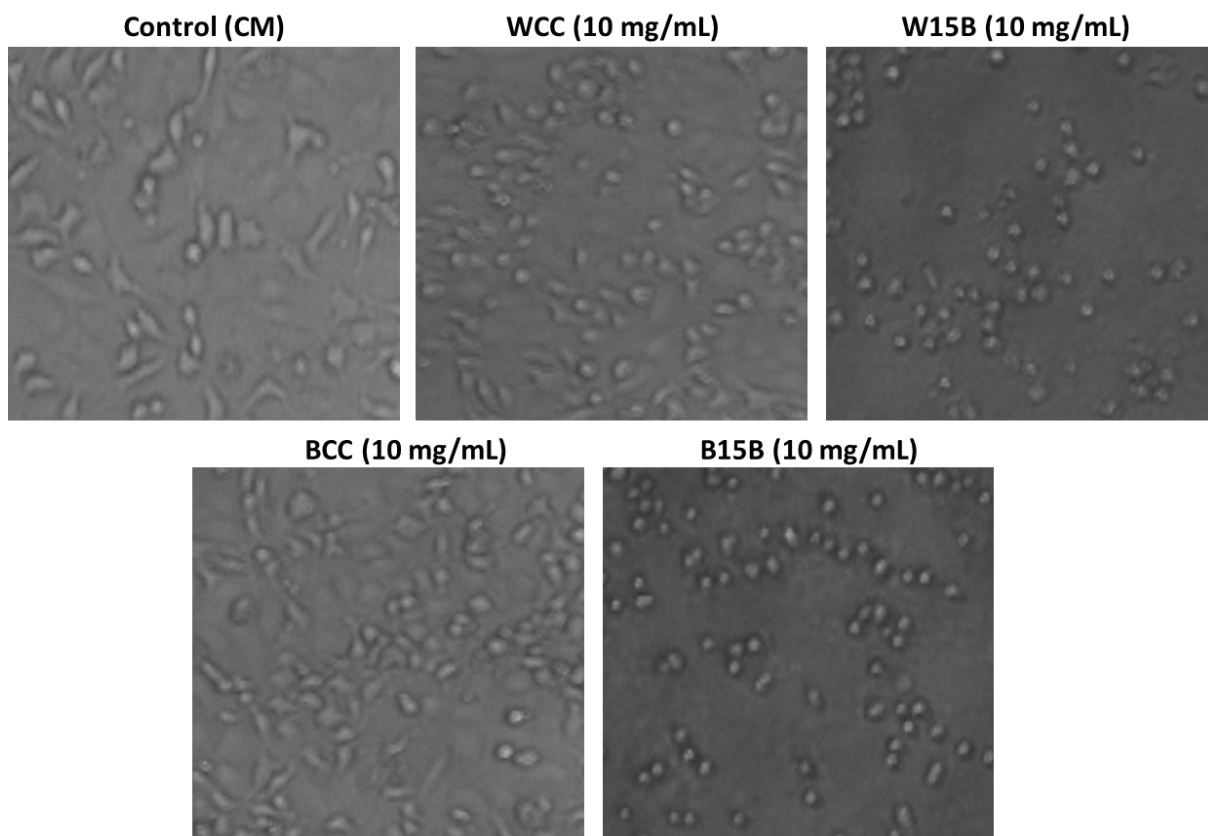


Figure 8.1. Anti-proliferative activity on HepG2 cell line (under microscope observation). Cells treated with the cookie digesta for 72 h
WCC- wholemeal wheat cookie control, W15B- wholemeal wheat + 15 % blackcurrant powder, BCC- wholemeal barley cookie control, B15B- wholemeal barley + 15 % blackcurrant powder

Table 8.4. Anti-proliferative activity and cytotoxicity of cookie digesta on HepG2 cell line

Anti-proliferative activity (EC ₅₀); Cytotoxicity (CC ₅₀)					
	EC ₅₀				CC ₅₀
Digestion Phase	WCC	W15B	BCC	B15B	All sample (mg/mL)
Oral	252.7 ± 11.23 ^a	38.38 ± 2.39 ^a	257.8 ± 9.88 ^a	46.53 ± 2.19 ^a	>200
Gastric	89.38 ± 9.17 ^b	30.69 ± 2.38 ^b	84.87 ± 7.59 ^b	33.47 ± 1.52 ^b	>200
Intestine	61.59 ± 4.82 ^c	7.648 ± 0.63 ^c	68.13 ± 2.33 ^c	6.88 ± 0.71 ^c	>120
Colon	58.92 ± 4.10 ^d	1.02 ± 0.11 ^d	61.92 ± 01.89 ^d	1.97 ± 0.08 ^d	>120

Data expressed as mg /mL sample, DW; *n* = 3, values with different letter in each column are significantly different (*p* <0.05)

8.3.5. Cellular antioxidant capacity, CAA on HepG2 cell model

Oxidative and/or pathological stresses are thought to be the leading cause of chronic diseases whereas antioxidants play a key role in cellular homeostasis (Valko et al. 2007). Chemical assay of antioxidant capacity of bioactive compounds might be dissimilar in complex biological systems, as they might not be working as individual entities to neutralize the radical. Biologically, CAA assay are

more relevant to physiological conditions, intracellular uptake, distribution and metabolism. To evaluate the antioxidant capacity of bioactive food digesta at the cell level, CAA assay was carried out with human liver cancer cell, HepG2, where DCFH-DA were used as probe with samples, and then ABAP were added to induced peroxy radical oxidization. Two protocols (“PBS wash” and “no PBS wash”) were followed to examine the cellular uptake of the compounds. Data is presented in Table 8.5, as micromole quercetin equivalent per 100 gram, DW.

Increased CAA values were observed with the no PBS wash treatment at the end of digestion for samples B15B (122.70 $\mu\text{mol QE}/100\text{ g, DW}$) and W15B (113.40 $\mu\text{mol QE}/100\text{ g, DW}$), this was at least two to three-fold higher than the control cookie. The PBS wash treatment of the same samples also exhibited higher CAA values at the same ratio. The results indicate the potential of blackcurrant to enhance the antioxidant ability of the cookies. Previous reports have indicated that the bioactive compounds quercetin and kaempferol available in blackcurrant act as a powerful antioxidant (Vagiri et al. 2013). In comparison with the TPC value of the different digestion phases and the trolox equivalent antioxidant assay were significantly ($P < 0.05$) higher than the CAA value in all samples. This indicates that some bioactive compounds could be available in the digesta which have powerful antioxidant ability in HepG2 cell model. The CAA values reflect the complex biological activity including absorption, metabolism and distribution, regardless of the scavenging free radicals of chemical assays. The PBS wash treatment CAA values were lower in the four digestion phases of all samples which compares with no PBS wash and is in agreement with other previous studies (Wolfe & Liu 2007; Guo et al. 2017). This could be due to the physico-chemical properties of compounds present in the digesta, including molecular size, solubility, polarity and their bioavailability at the cellular level (Holmström & Finkel 2014; Zhu et al. 2015).

Table 8.5. Cellular antioxidant activity (CAA) of cookie digesta

Digestion phase	WCC		W15B	
	No PBS wash	PBS wash	No PBS wash	PBS wash
Oral	10.53 ± 0.82 ^d	6.31 ± 0.18 ^d	17.79 ± 1.03 ^d	10.67 ± 0.14 ^d
Gastric	22.81 ± 1.77 ^c	14.83 ± 0.91 ^c	37.04 ± 2.61 ^c	21.48 ± 1.83 ^c
Intestine	33.76 ± 1.16 ^b	22.50 ± 1.10 ^b	80.81 ± 2.67 ^b	52.52 ± 2.02 ^b
Colon	41.57 ± 2.49 ^a	24.94 ± 3.97 ^a	113.40 ± 8.32 ^a	67.48 ± 4.90 ^a
Phase	BCC		B15B	
	No PBS wash	PBS wash	No PBS wash	PBS wash
Oral	11.09 ± 0.95 ^d	5.95 ± 0.22 ^d	17.59 ± 1.13 ^d	10.99 ± 0.74 ^d
Gastric	21.62 ± 1.02 ^c	15.13 ± 1.11 ^c	33.40 ± 2.06 ^c	20.46 ± 1.79 ^c
Intestine	28.24 ± 2.11 ^b	17.41 ± 1.38 ^b	87.64 ± 3.31 ^b	55.50 ± 5.02 ^b
Colon	42.34 ± 3.60 ^a	29.64 ± 2.35 ^a	122.70 ± 5.81 ^a	73.99 ± 6.63 ^a

Data expressed as $\mu\text{mol QE}/100\text{ g sample, DW}$; $n = 3$, values with different letter in each column are significantly different ($p < 0.05$)

WCC- wholemeal wheat cookie control, W15B- wholemeal wheat + 15 % blackcurrant powder, BCC- wholemeal barley cookie control, B15B- wholemeal barley + 15 % blackcurrant powder

8.3.6. IL-1B, IL-6, NF-kB and NUCB/Nesfatin 1 genes expression in HepG2 cell model

Proinflammatory cytokines, interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and nuclear transcription factor- kB (NF-kB) are thought to be important regulators of inflammation and development of chronic diseases. Upregulation of NF-kB target genes including IL-1 β and IL-6 genes expression have been associated with chronic inflammation and mediate cell proliferation (Blackwell & Christman 1997). Controlling the expression of these inflammatory genes activates the immune response and promotes immunity. The effects of cookie digesta on the relative expression of the three genes related inflammation were assessed by RT-qPCR to determine the biological effects of bioactive cookies in HepG2 cells. All samples exhibited downregulation of IL-1 β and IL-6 and NF-kB in digested samples (control vs tested samples) as shown in Figure 8.2. The downregulation pattern of these inflammatory genes was associated with an increased antioxidant value as reported in CAA analysis. The blackcurrant incorporation cookie digesta inhibited the expression of IL-6 and NF-kB genes in comparison with the control cookies. It is noteworthy that the inflammatory factor IL-1 β was highly downregulated when treated with samples W15B (-0.36 ± 0.06) and B15B (0.21 ± 0.01). The reason for the W15B and B15B treated samples showing regulation of gene expression may be due to the composition of the bioactive compounds, and their greater biological activity in a cell model, leading

to potential anti-inflammatory/anticancer effects and/or corresponding diseases (Valko et al. 2007; Iwata et al. 2017). Evidence suggests that upregulation of appetite regulator “nucleobindin-2/nesfatin-1 (NUCB/Nesfatin-1)” plays an important role in increasing satiety, glycaemic management and reducing body weight (Maejima et al. 2009). To determine the potential effects of bioactive model, the expression of NUCB/Nesfatin-1 was examined in HepG2 cells treated with digested samples. In the cell model, administration of the tested samples greatly influenced the expression of NUCB/Nesfatin-1 genes in all treatment (Figure 8.2). The relative expressions of NUCB/Nesfatin 1 in both blackcurrants containing cookie digesta were upregulated by about three-fold compared with the control cookies digesta, and ten to fifteen-fold compared to the control. These results indicate that the bioactive compounds present in the cookies are a prominent regulator of anorexigenic NUCB/Nesfatin 1 mRNA expression.

The bioactive polyphenols available in berries potentially inhibit the expression of inflammatory markers in animal and cell models (Kaume et al. 2012; Wang et al. 2017a). Previous studies have shown that the high phenolic content in berries potentially reduce pro-inflammatory cytokine IL-6, TNF- α (Overman et al. 2010; Wang et al. 2017a). The biological activities of different berry extracts have been investigated widely, however no research has shown so far that their potential activity after simulated digestion in the cellular level may be mediated synergistically by other compounds such as those contained in whole grains. The results presented in this research indicate that the biofunctional ingredients especially polyphenols, flavonoids and anthocyanins present in the blackcurrant incorporated cookie played an important role in regulation of certain genes expression, which may lead to a therapeutic effect on certain diseases. The results suggest a synergy between blackcurrant and whole grain bioactive compounds.

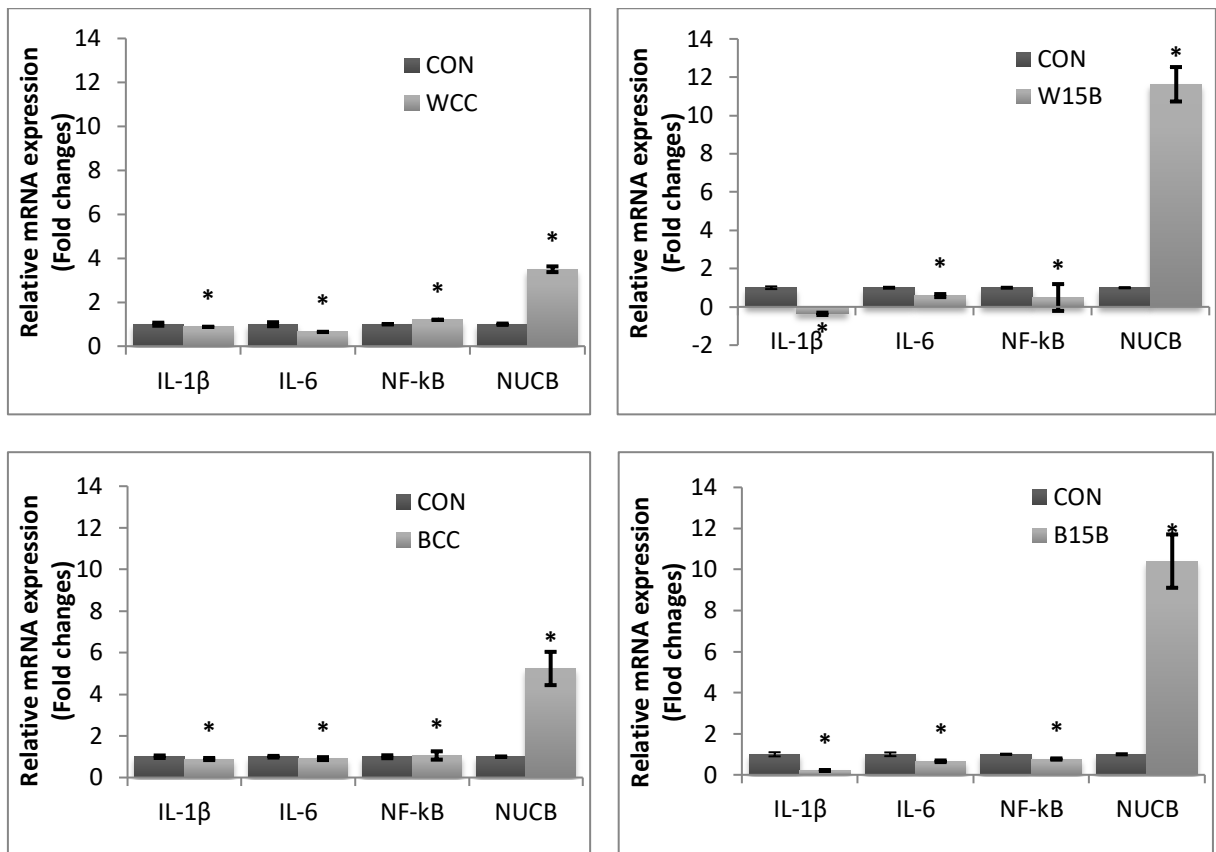


Figure 8.2: Relative mRNA expression (Fold change) of IL-1 β , IL-6, NF-kB and NUCB after treated with WCC, W15B, BCC and B15B cookie digesta (10 mg/mL) for 12 hours; Asterisk (*) are significantly different ($p < 0.05$) WCC- wholemeal wheat cookie control, W15B- wholemeal wheat + 15 % blackcurrant powder, BCC- wholemeal barley cookie control, B15B- wholemeal barley + 15 % blackcurrant powder

8.4. Conclusion

Biofunctional molecules from blackcurrant and whole grains synergistically contributed to the potential health benefits including improved cellular antioxidant activity, anti-proliferation and modulation of gene expression. Although the *in vivo* digestion and cellular environment are much more complex, the findings clearly show that the bioactive compounds-rich blackcurrant cookie potentially improved satiety and suppressed inflammatory cytokine regulation, which are relevant to the pathogenesis. The underlying mechanisms behind those potential effects should be further investigated on human and/or animal studies.

Chapter 9

The synergy of astaxanthin rich microalgae *Haematococcus pluvialis* with wholemeal cereals in a model food: a potential biological effect on HepG2 cell model

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Abstract

Haematococcus pluvialis microalgae is a natural source of astaxanthin. In this study we incorporated 15 % astaxanthin-rich *H. pluvialis* microalgae into a wholemeal wheat and barley flour cookie (as a model food) and then conducted a simulated digestion to determine phytochemical composition during digestion. Significantly ($p < 0.05$) higher TPC was found in the intestinal phase of digestion for the enriched cookies compared to the control. These were associated with high antioxidant values. Ferullic and cinnamic acids were identified as the most dominant phenolics via RP-HPLC. Astaxanthin incorporated cookie digesta significantly affected the inhibition of HepG2 cancer cell proliferation. The astaxanthin digesta also regulated the expression of NUCB2/nesfatin-1, inflammatory cytokines IL-1 β , IL-6 and NF-kB signalling. These results provide novel insights into food functional molecules.

9.1. Introduction

Recently there has been a trend to focus on microalgae as a part of human diet and its use in the functional food, pharmaceutical and nutraceutical industries. This interest sparks from the potential health-promoting effects of the bioactive compounds present in microalgae, such as carotenoids and other polyphenolic antioxidants, as well as the basic nutrients (Nair et al. 2015). Initially, microalgae were considered useful in human nutrition as a potential source of additional protein (Borowitzka 2013), however more recent research attention has turned to the high value of functional molecules found in microalgae.

Haematococcus pluvialis is single-cell green algae and processes high biologically active metabolites, most notably astaxanthin (1.5-3.0 %) (Lorenz & Cysewski 2000). Astaxanthin is a carotenoid considered a powerful antioxidant (several fold greater in activity than β -carotene and vitamin E) (Guerin et al. 2003; Ni et al. 2015). *H. pluvialis* contains significant amounts of micronutrients such as carotenoids, polyphenolic compounds, vitamins, minerals, fatty acids and phytosterols (Guerin et al. 2003) and has been shown to have potential in alleviating immunopathology, lipid peroxidation, insulin resistance, diet induced obesity and cancer (Ikeuchi et al. 2007; Nair et al. 2015; Ni et al. 2015). A number of prospective studies have showed that high consumption of antioxidant such as carotenoids lower the risk of major neurodegenerative diseases such as Alzheimer's, and age associated macular degeneration (Guerin et al. 2003). However, comparatively little emphasis has been laid on the specific biological contribution of properly processed microalgae to metabolic and health effects in cell and/or animal model.

Extensive studies have been conducted on cereal grains and their health effects, but limited research has demonstrated the impact of the metabolites of specific types of whole grain food in cellular response. Whole grains (milled intact grain including endosperm, germ and bran) such as wheat and barley contain a higher amount of bioactive components than refined cereal flours. This is partly due to higher levels of dietary fibre, vitamins, minerals and phenolics (Flight & Clifton 2006). In addition, whole grain food materials have been shown to exert a lower glycaemic index (20 -30 %) and higher biological potential using both *in-vitro* and *in-vivo* studies, possibly due to their bioactive compounds affects the metabolism by influencing the rate of digestion and absorption (Venn & Mann 2004).

Many of the current research manuscripts describe the functionality of food bioactive ingredients in relation to the antioxidant activity of food bioactive compounds based on chemical assays, commonly-used DPPH, ABTS and ORAC, but the numerous metabolic and physiological factors could attenuate the activity at cellular level. Therefore, in this study, we developed a model food (a cookie comprising of wholemeal wheat and barley) with incorporation of 15 % astaxanthin-rich microalgae powder to determine the biological effects on cell based models using HepG2, a human cancer cell model.

9.2. Materials and methods

9.2.1. Chemicals and reagents

Described in section 3.1.

9.2.2. Simulated multiphase enzymatic digestion

Described in section 3.7.2.

9.2.3. Determination of total phenolic content (TPC)

Described in section 3.8.

9.2.4. Major phenolic acids composition determined by RP-HPLC

Described in section 3.10.

9.2.5. Chemical antioxidant analysis by ORAC

Described in section 3.9.2.

9.2.6. Cell culture and culture condition

Described in section 3.18.

9.2.7. Cellular antioxidant analysis (CAA) of digesta

Described in section 3.19.

9.2.8. Cytotoxicity and anti-proliferative activity

Described in section 3.20.

9.2.9. Quantitative real-time PCR and regulation of gene expression

Described in section 3.21.

9.2.10. Statistical analysis

Described in section 3.24.

9.3. Result and discussion

9.3.1. The total phenolic content (TPC) and antioxidant capacity (ORAC) of digesta samples

Cookies were subjected to a simulated *in-vitro* digestion process (oral, gastric, intestinal and colon) and the digesta was recovered from each stage to determine comparisons for phytochemical properties. The total phenolic content of the digesta (TPC) is shown in Table 9.1. The TPC values were significantly ($P < 0.05$) increased during the gastrointestinal digestion, and the highest TPC were released after intestinal phase of all experimental cookies. This could be due to the complex food matrix of phenolics and protein, which are being released during protein digestion (Chandrasekara & Shahidi 2012). Cereal proteins have been shown to be digested at different rates in different physiological condition from upper to lower gastrointestinal tract, and this may have led to the attenuate the phenolics release (McGhie & Walton 2007). The 15 % astaxanthin incorporated wholemeal cookies (W15A and B15A) exerted about 50 % higher TPC in comparison with wheat and barley control cookie (WCC and BCC), indicating that the astaxanthin-rich microalgae *H. pluvialis* contain higher phenolic compounds (Hossain et al. 2017a). It is noteworthy that the colon phase digesta were exerted lower TPC value of all experimental cookies when compared with intestinal TPC, which is in agreement with previous studies (Bermúdez-Soto et al. 2007; Flores et al. 2014; Su et al. 2017). This could be due to the different pH condition affected phenolic stability (Bermúdez-Soto et al. 2007). Light, O_2 and other chemicals may degrade/oxidized the phenolics during simulated digestion (Farah et al. 2005). Moreover, interaction with enzymes and other food compounds such as starch-phenolics, amino acids of protein-phenolics and isomerization also manipulate the availability of phenolics during simulated digestion (Barros et al. 2012; Bordenave et al. 2014; Sun et al. 2018).

The total antioxidant capacity of each cookie digesta, at each digestion stage, was evaluated by ORAC assay and these results are presented in Table 9.2. The ORAC values significantly ($P < 0.05$) increased at the end of gastrointestinal digestion for all experimental cookies, associated with the higher TPC values already observed. The digesta from the astaxanthin incorporated cookies (W15A and B15A)

exhibited significantly ($p < 0.05$) higher antioxidant capacities compared to that of the control cookies (WCC and BCC) at all of the digestion stages. Astaxanthin has higher antioxidant properties than other phenolics such as carotenoids (Guerin et al. 2003). Astaxanthin-rich microalgae *H. pluvialis*, which contained higher bioactive compounds, might have acted individually and/or synergistically with wholemeal cereals and lead to the significant differences observed (Hamed et al. 2015; Nair et al. 2015).

Table 9.1. Total phenolic content of cookie digesta

Phase\Sample	WCC	W15A	BCC	B15A
Oral	44.37 ± 5.37 ^d	109.45 ± 0.39 ^c	41.04 ± 0.30 ^d	115.85 ± 4.66 ^c
Gastric	141.01 ± 4.79 ^c	302.49 ± 2.78 ^a	112.02 ± 3.32 ^c	269.52 ± 4.26 ^b
Intestine	211.28 ± 1.96 ^a	304.74 ± 4.30 ^a	183.50 ± 2.48 ^a	302.24 ± 1.99 ^a
Colon	193.7 ± 25.00 ^b	262.51 ± 2.67 ^b	162.32 ± 2.23 ^b	279.07 ± 3.40 ^b

Data expressed as MEAN mg GAE/100 g sample, $n = 3$; values with different letter in each column are significantly different ($p < 0.05$)

Table 9.2. Chemical antioxidant capacity (ORAC values) of cookie digesta

Phase	WCC	W15A	BCC	B15A
Oral	15.34 ± 3.93 ^d	24.18 ± 3.93 ^d	9.63 ± 2.9 ^d	21.83 ± 2.90 ^d
Gastric	53.76 ± 8.71 ^c	60.98 ± 3.92 ^c	40.12 ± 7.86 ^c	49.79 ± 3.38 ^c
Intestine	89.75 ± 2.56 ^a	99.40 ± 5.17 ^a	89.33 ± 3.54 ^a	101.58 ± 8.96 ^a
Colon	86.51 ± 6.57 ^b	95.59 ± 5.31 ^b	81.28 ± 10.84 ^b	95.52 ± 3.34 ^b

Data expressed as MEAN NetAUC Trolox equivalents ($\mu\text{mol TE/g}$ sample, DW); $n = 3$, values with different letter in each column are significantly different ($p < 0.05$)

9.3.2. Quantification of major phenolic compounds

HPLC analysis was performed on all digesta samples and chromatographic profiles were evaluated to quantify the major phenolic acids at each stage of digestion of the cookies. Results are presented in Table 9.3. Generally, the total number of peaks was consistent in same group of samples, however the peak areas varied greatly. This indicated that the concentration of phenolic acids varied at different digestion phase. Astaxanthin incorporated cookie digesta (W15A: 192.63 mg/100 g, DW and B15A: 167.51 mg/100 g, DW) were observed to exhibit the highest total phenolic acids in comparison with control cookie (WCC: 136.25 mg/100 g, DW and BCC: 104.95 mg/100 g, DW). With regards to the specific components, *p*-hydroxybenzoic, *p*-coumaric and ferulic acid were observed to be the most abundant phenolic acids among all experimental cookie digesta. Cinnamic and salicylic acids were detected in astaxanthin incorporated cookie digesta, indicating that *H. pluvialis* contributed to the total phenolic acids of cookies, which is in agreement with previous research (Safafar et al. 2015).

Digesta from the oral phase of cookie digestion exhibited lower phenolic acids among all the other digestion stages, possibly due to the very short residence time (Zhu et al. 2016). The highest concentrations of phenolic acids were observed in the intestinal stage of digestion. A slight loss of phenolic compounds were observed in the colon stage, possibly due to the unstable phenolic acids properties at different pH condition and interaction with other compounds, as described previously (Bermúdez-Soto et al. 2007; Barros et al. 2012; Bordenave et al. 2014; Su et al. 2017).

Table 9.3. HPLC analysis of major phenolic acids composition in model food digesta (mg/100 g DW)

Sample	Phase	p-HA	VA	SA	CA	p-CA	FA	CiA	SaA	Total PAs
WCC	Oral	5.39 ± 0.1	4.67 ± 0.5	9.27 ± 0.1	nd	1.7 ± 0.0	21.77 ± 1.3	nd	nd	42.8 ± 2.0
WCC	Gastric	8.46 ± 0.8	17.12 ± 1.1	14.61 ± 0.6	6.75 ± 0.1	7.83 ± 0.8	46.92 ± 2.3	nd	nd	101.69 ± 5.7
WCC	Intestine	16.89 ± 0.8	19.13 ± 2.7	17.42 ± 1.1	5.60 ± 0.1	19.70 ± 0.7	57.51 ± 2.8	nd	nd	136.25 ± 8.2
WCC	Colon	19.82 ± 0.4	19.54 ± 1.3	17.80 ± 0.7	4.72 ± 0.2	16.05 ± 1.3	48.16 ± 1.6	nd	nd	126.09 ± 5.5
W15A	Oral	11.88 ± 0.3	4.02 ± 0.1	7.08 ± 0.6	9.63 ± 0.4	5.19 ± 0.2	19.78 ± 0.9	9.13 ± 0.8	nd	66.71 ± 3.3
W15A	Gastric	17.22 ± 0.9	15.84 ± 0.6	12.96 ± 0.6	16.87 ± 0.6	12.96 ± 0.3	48.72 ± 1.8	12.16 ± 0.5	7.02 ± 0.3	143.75 ± 5.6
W15A	Intestine	21.01 ± 1.0	16.89 ± 0.6	15.21 ± 0.6	19.33 ± 1.0	24.31 ± 1.1	64.11 ± 1.7	19.88 ± 1.1	11.89 ± 0.6	192.63 ± 7.7
W15A	Colon	19.92 ± 0.8	17.11 ± 1.1	15.32 ± 0.3	18.62 ± 0.6	24.68 ± 1.3	53.13 ± 2.1	17.22 ± 0.8	12.51 ± 0.7	178.51 ± 7.7
BCC	Oral	8.35 ± 0.8	6.96 ± 0.9	nd	15.87 ± 0.6	2.96 ± 0.0	16.49 ± 0.6	nd	nd	50.63 ± 2.9
BCC	Gastric	12.66 ± 0.8	16.06 ± 0.8	10.71 ± 0.0	19.36 ± 0.8	11.09 ± 0.9	29.32 ± 1.5	nd	nd	99.2 ± 4.8
BCC	Intestine	12.78 ± 0.1	15.69 ± 0.1	6.21 ± 0.0	23.49 ± 0.2	19.23 ± 1.5	27.55 ± 0.9	nd	nd	104.95 ± 2.8
BCC	Colon	10.56 ± 0.2	20.70 ± 0.8	4.59 ± 0.1	16.19 ± 0.4	12.94 ± 0.9	28.12 ± 0.7	nd	nd	93.1 ± 3.1
B15A	Oral	12.49 ± 0.5	7.66 ± 0.3	nd	18.00 ± 0.6	6.32 ± 0.3	18.42 ± 1.0	8.17 ± 0.6	3.06 ± 0.1	74.12 ± 3.4
B15A	Gastric	16.79 ± 0.8	15.72 ± 0.6	8.39 ± 0.3	24.23 ± 1.1	17.65 ± 0.9	35.11 ± 1.6	13.11 ± 0.9	6.02 ± 0.2	137.02 ± 6.4
B15A	Intestine	18.64 ± 1.1	16.03 ± 1.1	7.26 ± 0.7	29.35 ± 1.2	25.38 ± 1.1	38.21 ± 2.1	18.73 ± 0.4	13.91 ± 0.4	167.51 ± 8.1
B15A	Colon	20.13 ± 0.9	17.64 ± 0.9	7.11 ± 0.6	26.72 ± 1.0	19.84 ± 1.0	30.41 ± 2.2	17.38 ± 0.7	11.08 ± 0.5	150.31 ± 7.8

Data expressed as mg phenolic acid (PA) /100 g sample, DW; *n* = 3, values with different letter in each column are significantly different (*p* < 0.05)

Compounds are presented as: p-HA: p-hydroxybenzoic acid; VA: vanilic acid; SA: syringic acid; CA: caffeic acid, p-CA: p-coumaric acid, FA: ferulic acid; CiA: cinnamic acid; SaA: salicylic acid; PAs: phenolic acids

9.3.3. Cytotoxicity and inhibition of cell proliferation

The cytotoxic effects of each stage of digestion for the cookies were evaluated against HepG2 cell line in order to determine the toxicological activities of inclusion of astaxanthin. The CC_{50} values are presented in Table 9.4. At the oral and gastric stages of digestion the CC_{50} values were up to 200 mg/mL, whilst at the intestinal and colon stages the values were up to 120 mg/mL of all experimental cookies. The reactions appeared to be dose-dependent. This could be due to the enzymes, pancreatic bile salts used in the simulated digestion and/or bioactive metabolites in the system, have been reported previously (Zhu et al. 2015; Martins et al. 2016).

HepG2 cells were treated with non-toxic concentrations of each phase cookie digesta for 72 h to evaluate the anti-proliferation activity. The results are presented as median effective dose (EC_{50}), where lower (EC_{50}) value indicates higher efficacy. Generally, the lower gastrointestinal stage of digestion resulted in significantly stronger inhibition of HepG2 cell proliferation than the upper gastrointestinal stages in all experimental cookies (Table 9.4). This could be due to the potent bioactive compounds released in lower phase digestion, exerted significant anti-proliferative effects (Iwata et al. 2017). As shown in Figure 9.1, digesta from astaxanthin incorporated cookies inhibited the HepG2 cancer cell proliferation in comparison with wholemeal cookie control. The lowest EC_{50} value was observed in B15A digesta from the colon stage (5.98 mg/mL), followed by W15A (6.12 mg/mL), WCC (58.92 mg/mL) and BCC (61.92 mg/mL) respectively. Astaxanthin incorporated cookie digesta might release invasive bioactive compounds during simulated digestion, which have higher bioavailability and powerful anti-proliferation properties against HepG2 cells (Guerin et al. 2003; Nagaraj et al. 2012).

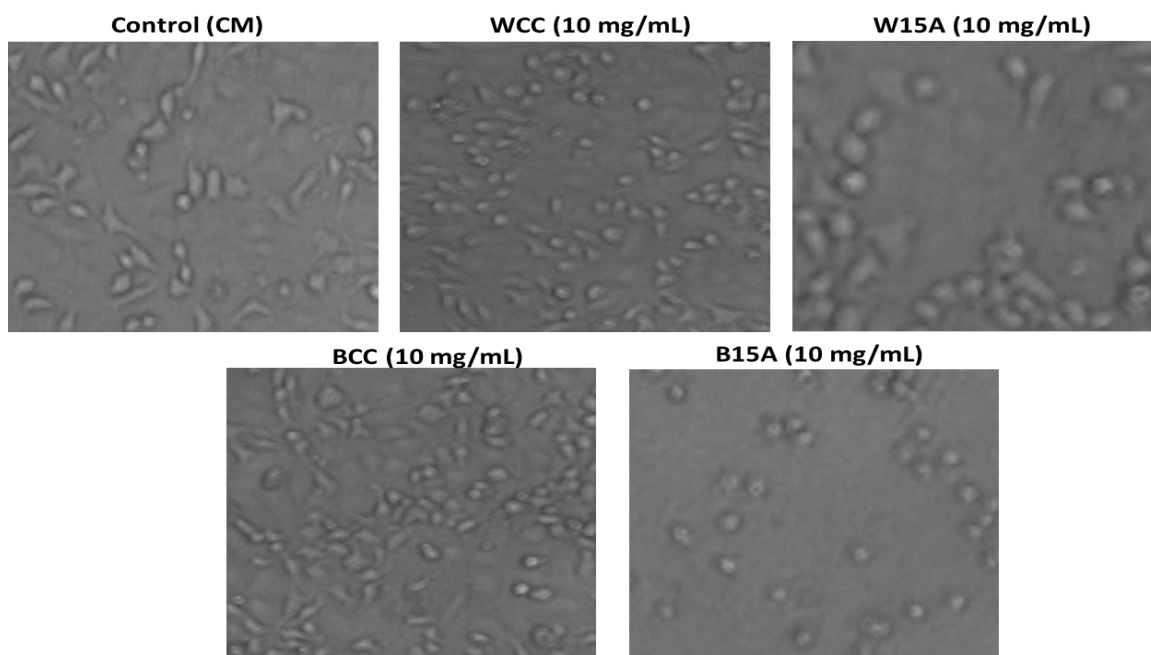


Figure 9.1. Anti-proliferative activity on HepG2 cell line (under microscope observation). Cells treated with the cookie digesta for 72 h

Table 9.4. Anti-proliferative activity and cytotoxicity of cookie digesta on HepG2 cell line
Anti-proliferative activity (EC_{50}); Cytotoxicity (CC_{50})

Phase	EC_{50}				CC_{50}
	WCC	W15A	BCC	B15A	All sample
Oral	252.7 ± 11.23 ^a	57.91 ± 3.11 ^a	257.8 ± 9.88 ^a	55.34 ± 2.67 ^a	>200
Gastric	89.38 ± 9.17 ^b	32.80 ± 2.56 ^b	84.87 ± 7.59 ^b	33.58 ± 2.07 ^b	>200
Intestine	61.59 ± 4.82 ^c	14.29 ± 1.04 ^c	68.13 ± 2.33 ^c	15.71 ± 1.11 ^c	>120
Colon	58.92 ± 4.10 ^d	6.12 ± 0.84 ^d	61.92 ± 01.89 ^d	5.98 ± 0.71 ^d	>120

Data expressed as mg /mL sample, DW; $n = 3$, values with different letter in each column are significantly different ($p < 0.05$)

WCC- wholemeal wheat cookie control, W15A- wholemeal wheat + 15 % astaxanthin powder, BCC- wholemeal barley cookie control, B15A- wholemeal barley + 15 % astaxanthin powder

9.3.4. Cellular antioxidant activity (CAA)

Oxidative stress contributes to the pathogenesis of major chronic diseases, whereas antioxidants play a potential role in cellular homeostasis (Sheykhansari et al. 2018). The CAA assay is relevant to biological systems including cellular uptake and metabolism in the human body. The calculated CAA values for both “no PBS wash” and “PBS wash” are summarised in Table 9.5. Generally the higher the CAA value the stronger antioxidant capacity to the sample. The highest antioxidant capacities were observed in the digesta of astaxanthin incorporated cookie B15A (77.85 $\mu\text{mol QE/ 100 g}$ sample, DW), followed by W15A (72.19 $\mu\text{mol QE/ 100 g}$ sample, DW), then BCC (42.34 $\mu\text{mol QE/ 100 g}$

sample, DW) and WCC (41.57 $\mu\text{mol QE/ 100 g sample, DW}$) of no PBS wash protocol. This implies that the molecules released from astaxanthin incorporated cookies during simulated digestion, enhanced binding to the cell membrane and improved antioxidant capacity individually and/or synergistic manner (Geng et al. 2016). The PBS wash reflected the cell permeability of the molecules and showed comparatively low CAA value than no PBS wash protocol. Moreover, this could be proportionate to the solubility, polarity and intracellular bioactivity of the molecules (Zhu et al. 2015; Geng et al. 2016).

Table 9.5. Cellular antioxidant activity (CAA) of each phase cookie digesta

Phase	WCC		W15A	
	No PBS wash	PBS wash	No PBS wash	PBS wash
Oral	10.53 \pm 0.82 ^d	6.31 \pm 0.18 ^d	13.88 \pm 0.91 ^d	9.07 \pm 0.62 ^d
Gastric	22.81 \pm 1.77 ^c	14.83 \pm 0.91 ^c	29.61 \pm 1.44 ^c	18.43 \pm 1.21 ^c
Intestine	33.76 \pm 1.16 ^b	22.50 \pm 1.10 ^b	56.37 \pm 2.01 ^b	39.01 \pm 1.86 ^b
Colon	41.57 \pm 2.49 ^a	24.94 \pm 3.97 ^a	72.19 \pm 3.82 ^a	47.26 \pm 2.79 ^a
Phase	BCC		B15A	
	No PBS wash	PBS wash	No PBS wash	PBS wash
Oral	11.09 \pm 0.95 ^d	5.95 \pm 0.22 ^d	14.61 \pm 1.07 ^d	8.76 \pm 0.59 ^d
Gastric	21.62 \pm 1.02 ^c	15.13 \pm 1.11 ^c	30.21 \pm 2.56 ^c	19.11 \pm 1.51 ^c
Intestine	28.24 \pm 2.11 ^b	17.41 \pm 1.38 ^b	39.38 \pm 2.65 ^b	36.07 \pm 2.29 ^b
Colon	42.34 \pm 3.60 ^a	29.64 \pm 2.35 ^a	77.85 \pm 4.12 ^a	51.74 \pm 3.01 ^a

Data expressed as $\mu\text{mol QE/ 100 g sample, DW}$; $n = 3$, values with different letter in each column are significantly different ($p < 0.05$)

9.3.5. IL-1 β , IL-6, NF-kB and NUCB/Nesfatin 1 genes expression in HepG2 cell model

Overexpression of pro-inflammatory cytokines, particularly IL-1 β , IL-6 and transcription factors NF-kB have been linked to the abnormal cellular immunity and pathogenesis of major chronic diseases (Borsini et al. 2015). Controlling the regulation of these inflammation-mediating genes promote immunity. This chapter illustrates the relative expression of IL-1 β , IL-6 and transcription factors NF-kB assessed by RT-qPCR to evaluate the bioactivity cookie digesta in HepG2 cell model. All cookie digesta exhibited down-regulation of IL-1 β , IL-6 and nuclear factor NF-kB when compared with controls, Figure 9.2. Surprisingly, astaxanthin incorporated cookie digesta demonstrated significantly ($P < 0.05$) down regulation of inflammation related gene expression. This action might be mediated by their higher phenolics, which exerted significantly higher bioactivity chemically (ORAC assay) and at cellular level (CAA assay), similar to previous reports (Bae et al. 2015). Indeed, previous studies have illustrated that astaxanthin suppressed the inflammatory cytokine and NF-kB mediated

inflammation in high-fat diet fed mice (Huang et al. 2015). However, *H. pluvialis* traditionally known for their powerful antioxidant source but also shown to modify inflammatory pathway, decreased IL-1 β and IL-6 level in cell model.

Up-regulation of anorexigenic gene nucleobindin-2/nesfatin-1 (NUCB/Nesfatin-1) is involved in the regulation of gastrointestinal functions, food intake, satiety and metabolic homeostasis (Stengel 2015). Genetic analyses of cookie digesta, cells were treated 10 mg/mL of the digesta for 12 hours to determine the bioactivity in regulation of NUCB/Nesfatin-1 gene expression. The relative expression of NUCB/Nesfatin-1 gene was significantly ($P < 0.05$) up-regulated (about five-folds) in presence of astaxanthin incorporated cookie digesta when compared with controls, Figure 9.2. Previous studies suggested that NUCB/Nesfatin-1 levels influenced by circulating bioactive metabolites, tended to be linked with higher bioactive compounds (Rodriguez et al. 2017). However, genetic investigation of this study suggesting that combination of *H. pluvialis* incorporation in wholemeal cookie could be the part therapy of metabolic disorder.

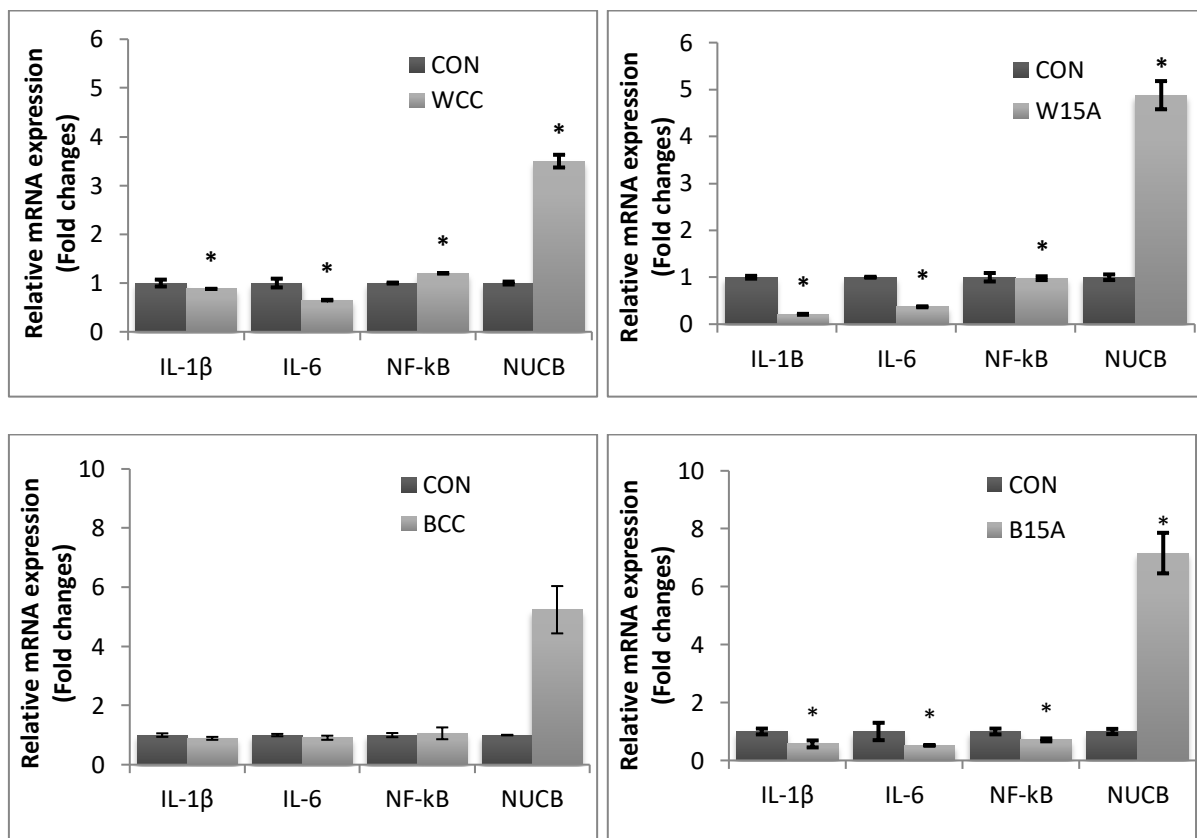


Figure 9.2. Relative mRNA expression (Fold change) of IL-1 β , IL-6, NF-kB and NUCB after treated with WCC, W15A, BCC and B15A cookie digesta (10 mg/mL) for 12 hours. Asterisk (*) are significantly different ($p < 0.05$) WCC- wholemeal wheat cookie control, W15A- wholemeal wheat + 15 % astaxanthin powder, BCC- wholemeal barley cookie control, B15A- wholemeal barley + 15 % astaxanthin powder

9.4. Conclusion

This study demonstrated the metabolic and physiological responses linked with different dietary compounds individually and/or synergistic manner. Astaxanthin inclusion in wholemeal wheat and barley cookies influenced the richness of bioactive compounds, particularly phenolics and beneficial antioxidant functions. Furthermore, genetic modulation that appears to benefits from digesta suggested new insight of food functionality.

Chapter 10

Cellular biological activity and regulation of gene expression of antioxidant dietary fibre isolated from blackcurrant incorporated in the wholemeal cereal cookies

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Abstract

Free and bound phenolics were extracted from the fibre fraction of wholemeal (W) wheat and barley (B) cookies which had been fortified with 15 % blackcurrant powder. Blackcurrant enriched cookies contained between 55 - 66 % higher total phenolics respectively compared control cookies. Ferulic acid in wheat and barley cookie extracts, and quercetin and kaemferol-3-glucoside in wheat cookies with 15 % blackcurrant were the dominant phenolic acids. Cellular antioxidant activity (CAA) was higher in samples with blackcurrant inclusion when evaluated in a cancer cell HepG2 model. Inhibition of cell proliferation was lower for the phenolic samples from cookies with blackcurrant addition. These samples suppressed the regulation of inflammatory cytokine IL-1 β (about 3 to 4-fold), IL-6 (about 2-fold) and transcription signalling factor NF-kB (about 2-fold) and showed an up-regulation of the satiety gene NUCB-2/Nesfatin-1 (about 4-fold) in compared with control samples.

10.1. Introduction

There is a great interest in the biologically active compounds within a diet which can help in the reduction of the risk of chronic diseases including all forms of cancers. Dietary bioactive compounds include polyphenol phytochemicals widely occurring in plant foods (fruits, vegetables and wholegrain cereals). Polyphenols, including phenolic acids and flavonoids, may act as antioxidants and protect health from diseases through a number of mechanisms, particularly the elimination of free radicals and regeneration of other antioxidants such as vitamin E. Accumulating evidence has shown that bioactive compounds including phenolic acids, flavonoids and polysaccharides are inversely

associated with major chronic diseases such as obesity (Nelson et al. 2016), diabetes (Sampath et al. 2017), cardiovascular disease (Dalgård et al. 2008), neurodegenerative diseases (Rodriguez et al. 2017) and cancer (Amatori et al. 2016; Nelson et al. 2016). Cumulative oxidative stress (elevated level of reactive oxygen species, ROS, free radicals) is the aetiology of these chronic diseases, whilst bioactive compounds particularly phenolics has been documented to potentially attenuate the free radicals.

Blackcurrant berry (*Ribes nigrum*) is widely available in many countries including New Zealand. The health promoting properties of blackcurrant have been studied during in relation to their biological function and nutritional value (Lehtonen et al. 2011). Numerous studies have demonstrated that the consumption of blackcurrant berry may modulate metabolic diseases and enhance cognitive functionalities (Esposito et al. 2015; Watson et al. 2015). Accumulating data have demonstrated in recent studies that aggregated bioactive compounds such as polysaccharides, phenolics, flavonoids and carotenoids (mainly found in the outer regions of the berry fruit) are associated with disease prevention, which may not be readily hydrolysed by the gastrointestinal enzymes (Ayoub et al. 2016; Sumczynski et al. 2016). If these bioactive compounds are released and absorbed, their full potential conjugates may enhance protective health benefits.

Basic nutrition (such as carbohydrate and protein) aside, wholegrain cereals such as wheat and barley are excellent source of biologically active substances dietary fibre, phenolic acids, linoleic acids, vitamin E and minerals concentrated in the cell walls. There is mounting evidence supporting claims that higher consumption of wholegrain cereal foods significantly reduces the risk of metabolic diseases (Steemburgo et al. 2009; Nelson et al. 2016). Processing (such as refined grains) substantially reduces the content of these functional molecules. However, bioactive compounds may exist in free and bound forms in the plant foods. Most of the fruits and wholegrain cereals bioactive compounds (65 - 75 % of total polyphenols in wholegrain cereals) may be present in bound form to the cell wall materials (Adom & Liu 2002).

We have previously investigated the synergistic effects of blackcurrant incorporation in the wholemeal cereals model food and demonstrated a significant decrease in glucose release during *in-*

vitro enzymatic digestion (Hossain et al. 2017b). The current study evaluates the functional potential of such material in cellular antioxidant and anti-proliferative effects in human liver cancer cell lines, HepG2.

10.2. Materials and methods

10.2.1. Chemicals and reagents

The chemicals and reagents used for this study were described in section 3.1.

10.2.2. Total dietary fibre (TDF) isolation from cookies

Total dietary fibre (TDF) isolation from model food was carried out as described in section 3.12.

10.2.3. Free and bound phenolics extraction from TDF

The free and bound phenolics extraction from TDF were carried out as described in section 3.8.1 and 3.8.2.

10.2.4. Total phenolic content determination

The total phenolic content of free and bound phenolics extract were determined as described in section 3.8.

10.2.5. Chemical antioxidant analysis by ORAC

Described in section 3.9.2.

10.2.6. Major phenolic acids composition determined by RP-HPLC

Described in section 3.10.

10.2.7. Cell culture and culture condition

Described in section 3.18.

10.2.8. Cellular antioxidant analysis (CAA) of antioxidant-DF extracts

Described in section 3.19.

10.2.9. Cytotoxicity and anti-proliferative activity

Described in section 3.20.

10.2.10. Quantitative real-time PCR and regulation of gene expression

Described in section 3.21.

10.2.11. Statistical analysis

Described in section 3.24.

10.3. Result and discussion

10.3.1. Total phenolic content (TPC) of antioxidant-DF fractions

The phenolic extracts (free, bound and total fraction) of the four samples (WCC, W15B, BCC and B15B) were used to determine the TPC (expressed as gallic acid equivalents) as presented in Table 10.1. Significantly ($p < 0.05$) higher TPC values were observed in the free, bound and total phenolic fractions of samples W15B (about 55 % of WCC) and B15B (about 66 % of BCC) compared to their control samples (samples without incorporated with 15 % blackcurrant). These differences can be due to the higher bioactive polyphenols of blackcurrant (Kapasakalidis et al. 2006). The bound phenolics of the samples were higher than the free phenolics concentration, which is in agreement with previous studies (Sosulski et al. 1982; Vitaglione et al. 2008; Acosta-Estrada et al. 2014).

Table 10.1. Free, bound and total phenolic content of antioxidant-DF extract

Sample	Free phenolics	Bound phenolics	Total phenolics
WCC	191.88 ± 6.86 ^c	308.62 ± 3.10 ^c	500.5 ± 9.96 ^c
W15B	327.73 ± 9.48 ^a	451.16 ± 8.08 ^a	778.89 ± 17.56 ^a
BCC	148.47 ± 6.10 ^d	264.52 ± 5.99 ^d	412.99 ± 12.09 ^d
B15B	325.53 ± 7.18 ^b	360.93 ± 7.11 ^b	686.46 ± 14.29 ^b

Data expressed as mg GAE/100 g sample, $n = 3$; values with different letter in each column are significantly different ($p < 0.05$)

WCC- wholemeal wheat cookie control, W15B- wholemeal wheat + 15 % blackcurrant powder, BCC- wholemeal barley cookie control, B15B- wholemeal barley + 15 % blackcurrant powder

10.3.2. Oxygen radical absorbance capacity (ORAC) antioxidant assay

Bioactive phenolics are reportedly primary antioxidant molecules with powerful free radical scavenging capacity, which acts as a protective agent in the human body (Pan et al. 2017). Antioxidant capacity of phenolic extracts was demonstrated *in-vitro* (Table 10.2). Phenolic extracts from W15B and B15B samples exhibited a significantly ($p < 0.05$) higher Trolox equivalent ($\mu\text{mol TE/g}$ sample) antioxidant capacity (W15B 13 % higher than WCC; B15B 70 % than BCC). The higher

phenolic contents of the samples may confer protection against free radicals and potent antioxidant ability. The higher antioxidant capacity is might be due to their redox properties and chemical structure of the phenolic compounds available in the blackcurrant incorporated antioxidant-DF fractions (Heim et al. 2002). Not all phenolic compounds show similar antioxidant abilities (Tabart et al. 2009). The total biological activity might be attributed to the individual molecule present in the extract, structural characteristics and their synergistic effects (Rice-Evans et al. 1996; Sampath et al. 2017). Therefore, the compositional analysis of the phenolic extracts was further evaluated.

Table 10.2. Extracellular antioxidant activities (ORAC values) of antioxidant-DF fractions

Sample	Free	Bound	Total
WCC	73.11 ± 4.46 ^{ab}	100.68 ± 3.27 ^a	173.79 ± 7.73 ^b
W15B	78.74 ± 4.38 ^a	117.73 ± 5.65 ^a	196.47 ± 10.03 ^a
BCC	28.42 ± 2.67 ^c	57.32 ± 3.49 ^c	85.74 ± 6.16 ^d
B15B	64.34 ± 2.89 ^b	81.83 ± 1.04 ^b	146.17 ± 3.93 ^c

Data expressed as Trolox equivalents ($\mu\text{mol TE/g sample, DW}$); $n = 3$, values with different letter in each column are significantly different ($p < 0.05$)

10.3.3. Evaluation of major phenolic acids

It has been well documented that phenolic compounds may act as antioxidants and protect human body (Rice-Evans et al. 1996; Balasundram et al. 2006). Therefore, it is important to determine the phenolic profile of the antioxidant-DF extracts to evaluate their potential biological activity at cellular level. A total of ten major phenolic compounds were quantified in the cookie samples (Table 10.3). The clustering of total phenolics, W15B and B15B were showed the specific phenolic profile compared to WCC and BCC. *p*-hydroxybenzoic, syringic acid, *p*-coumaric acid, ferrulic acids, are major phenolic compounds in extracts WCC and BCC. Quercetin, quercetin-3-rutinoside, kaemferol-3-glucoside, catechin were higher in extracts from W15B and B15B tested samples. These have previously been identified in blackcurrant berry material (Sójka et al. 2009; Mäkilä et al. 2017). These bioactive compounds, especially quercetin derivatives and kaemferol-3-glucoside, have been identified as a potent antioxidant (Roleira et al. 2015; Srivastava et al. 2016). Therefore, the potential antioxidant capacity (chemically and at cellular level) and biological activity might be related to these phenolic compounds.

Table 10.3. Major phenolic acids of different antioxidant-DF extract

Sample	Fraction	p-HA	VA	SA	CA	p-CA	FA	Q-3-R	Q	K-3-G	CA _t
WCC	Free	7.82 ± 0.09	8.94 ± 0.48	11.79 ± 0.71	13.01 ± 0.47	9.67 ± 0.81	9.30 ± 0.75	nd	nd	nd	nd
WCC	Bound	15.33 ± 0.54	6.77 ± 0.52	20.98 ± 0.12	10.01 ± 0.74	10.73 ± 0.06	6.50 ± 0.06	nd	nd	nd	nd
WCC	Total	23.15 ± 0.63	15.72 ± 1.00	32.78 ± 0.83	23.03 ± 1.21	20.41 ± 0.87	15.80 ± 0.81	nd	nd	nd	nd
W15B	Free	16.05 ± 0.47	5.86 ± 0.04	13.64 ± 0.14	9.25 ± 0.48	10.97 ± 0.17	10.71 ± 0.71	25.26 ± 1.32	13.71 ± 0.05	11.61 ± 0.11	4.45 ± 0.05
W15B	Bound	20.06 ± 0.83	6.38 ± 0.13	18.97 ± 0.78	9.72 ± 0.05	14.37 ± 0.58	11.57 ± 0.07	44.46 ± 1.48	28.93 ± 0.67	16.14 ± 0.29	5.35 ± 0.06
W15B	Total	36.12 ± 1.30	12.25 ± 0.17	32.62 ± 0.92	18.97 ± 0.53	25.35 ± 0.75	22.29 ± 0.78	69.73 ± 2.80	42.65 ± 0.72	27.76 ± 0.40	9.81 ± 0.11
BCC	Free	10.81 ± 0.66	2.51 ± 0.02	nd	12.75 ± 0.55	9.94 ± 0.41	16.07 ± 1.58	nd	nd	nd	nd
BCC	Bound	21.52 ± 0.64	2.61 ± 0.03	nd	21.56 ± 0.54	10.14 ± 0.09	19.12 ± 0.68	nd	nd	nd	nd
BCC	Total	32.34 ± 1.30	5.13 ± 0.05	nd	34.32 ± 1.09	20.09 ± 0.50	35.20 ± 2.26	nd	nd	nd	nd
B15B	Free	14.69 ± 0.83	16.03 ± 0.05	nd	9.36 ± 0.62	13.72 ± 0.48	12.90 ± 0.65	21.23 ± 1.05	15.22 ± 0.53	5.85 ± 0.45	2.03 ± 0.01
B15B	Bound	17.92 ± 0.24	22.44 ± 0.67	nd	10.86 ± 0.19	19.76 ± 0.52	16.77 ± 0.14	29.06 ± 0.99	28.78 ± 0.88	9.31 ± 0.06	5.28 ± 0.04
B15B	Total	32.62 ± 1.07	38.48 ± 0.72	nd	20.23 ± 0.81	33.49 ± 1.00	29.67 ± 0.79	50.29 ± 2.04	44.00 ± 1.41	15.17 ± 0.51	7.31 ± 0.05

Data expressed as mg phenolic acid (PA) /100 g sample, DW; $n = 3$, values with different letter in each column are significantly different ($p < 0.05$)

Compounds are presented as: p-HA: p-hydroxybenzoic acid; VA: Vanilic acid; SA: syringic acid; CA: catechin, p-CA: p-coumaric acid, FA: ferulic acid; Q-3-R: quercetin-3-rutinoside; Q: quercetin; K-3-G: kaempferol-3-glucoside

10.3.4. Cellular antioxidant activity (CAA)

CAA assay reflects the more relevant to complex biological system including absorption, distribution and metabolism of the molecules, and physiological radical-scavenging/antioxidation mechanism at the cellular level. In our study, biologically relevant CAA assay were performed to investigate the intracellular antioxidant activity of the free and bound phenolic extracts respectively, which might be represents better accuracy than chemical antioxidant assays. The phenolic extracts of the antioxidant-DF were applied on human liver cancer cell model, HepG2. Our results demonstrated that the bioactive molecules of extracts have the ability to be absorbed by the cells and exerted potential antioxidant activity (Table 10.4).

The exogenous peroxy radical ABAP oxidizes DCFH to fluorescent DCF. The DCFH oxidation was inhibited by the all tested samples in a dose-dependent manner. A higher CAA value indicates greater cellular antioxidant capacity. Both the PBS wash and No PBS wash, W15B and B15B samples demonstrated significantly higher

CAA values than the WCC and BCC samples (W15B PBS wash 1141.29 ± 29.5 , No PBS wash 535.67 ± 9.06 $\mu\text{mol QE}/100$ g sample, DW; B15B PBS wash 1036.24 ± 26.60 , No PBS wash 493.41 ± 14.92 $\mu\text{mol QE}/100$ g sample, DW). The no PBS wash protocol exhibited higher CAA value, which in agreement with previous studies (Zhu et al. 2016; Guo et al. 2017). The cellular antioxidant ability mainly depends on the compounds solubility, cell permeability and physiological conditions (Wolfe & Liu 2007; Seiquer et al. 2015).

Table 10.4. Cellular antioxidant activity (CAA) of antioxidant-DF (free, bound and total fractions)

Sample	Free		Bound		Total	
	No PBS wash	PBS wash	No PBS wash	PBS wash	No PBS wash	PBS wash
WCC	302.74 ± 11.49^c	152.90 ± 8.43^c	435.94 ± 10.44^c	243.54 ± 8.99^c	738.68 ± 21.93^c	396.44 ± 17.42^c
W15B	526.74 ± 16.01^a	241.62 ± 6.09^a	614.55 ± 13.49^a	294.04 ± 2.97^a	1141.29 ± 29.50^a	535.67 ± 9.06^a
BCC	127.38 ± 6.92^d	60.08 ± 3.91^d	226.98 ± 7.20^d	101.78 ± 2.73^d	354.37 ± 14.12^d	161.87 ± 6.64^d
B15B	499.44 ± 12.31^b	215.27 ± 7.67^b	536.80 ± 14.29^b	278.13 ± 7.25^b	1036.24 ± 26.60^b	493.41 ± 14.92^b

Data expressed as $\mu\text{mol QE}/100$ g sample, DW); $n = 3$, values with different letter in each column are significantly different ($p < 0.05$)

10.3.5. Proliferative inhibition and cytotoxicity of antioxidant-DF

Abnormal cell growth and/or proliferation are key alterations in the pathophysiology of cancerous conditions. In order to investigate the effects of antioxidant-DF on cell proliferation, the human liver cancer cell lines, HepG2 were treated with different concentrations of samples for 72 h. The results indicated that the antioxidant-DF components significantly reduced the viability of the HepG2 cells in a concentration and time-dependent manner (5 to 160 mg/mL, 24 h to 72 h) as illustrated in Table 10.5. The bound fractions of the antioxidant-DF components exhibited significantly ($p < 0.05$) higher growth inhibition (lower EC_{50} value) than the free phenolic fractions. Samples from W15B and B15B had the highest anti-proliferative effects in both free and bound phenolic fractions, which could be due to the synergistic effects of the phenolic compounds (Srivastava et al. 2016). In addition, samples from W15B and B15B contained the phenolic compounds quercetin and catechin which have been reported to have high growth inhibition activity (Srivastava et al. 2016). The potential impact of bioactive phenolics on the significant reduction on cell growth treated with the phenolic extracts may

be related to the fact that the extracts had the ability to be absorbed by the cells, and this was observed even under lower concentration Figure 10.1. The inhibition of cell proliferation results of phenolic extracts supports previous studies indicating that higher affinity of bioactive molecules and intracellular mechanisms were associated with regulation effects of cell cycle and the induction of apoptosis (Bakowska-Barczak et al. 2009; Kosmala et al. 2014). An important consideration that there were no considerable toxic effects observed in the HepG2 cells at the applied dose of tested samples.

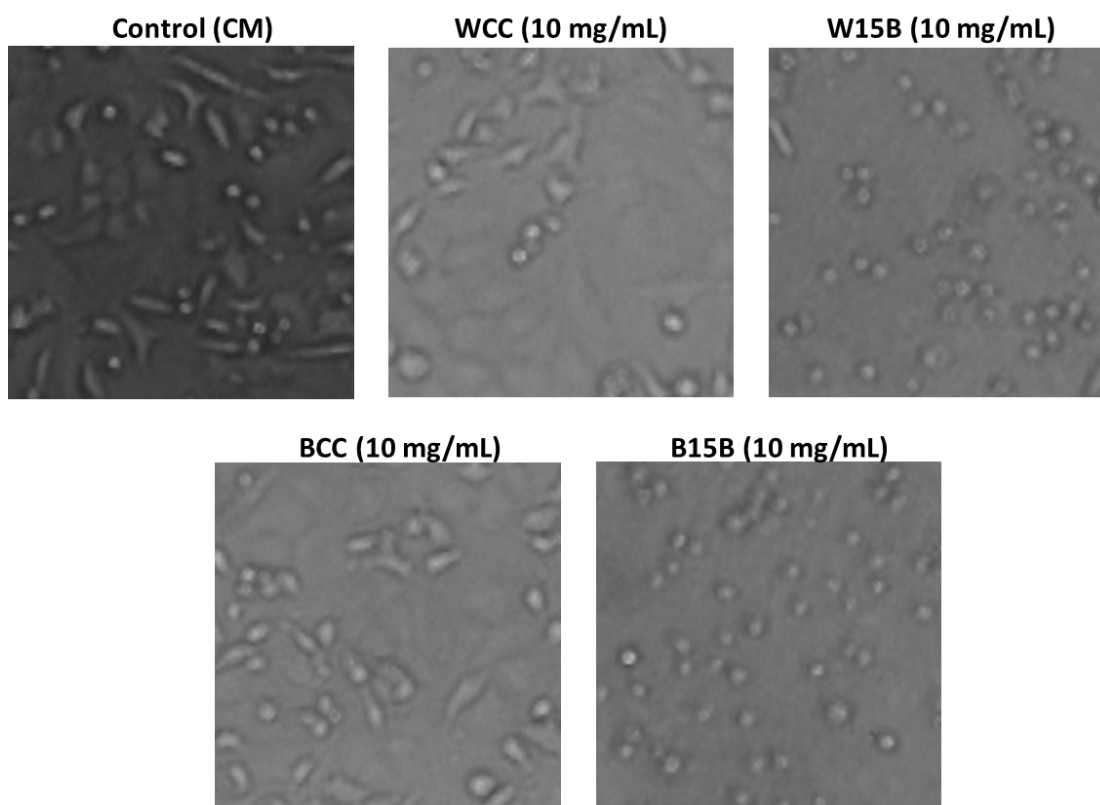


Figure 10.1. Anti-proliferative activity on HepG2 cell line (under microscope observation). Cells treated with antioxidant-DF for 72 h

Table 10.5. Anti-proliferative activity and cytotoxicity of antioxidant-DF fraction on HepG2 cell line

Sample	Anti-proliferation EC ₅₀ (mg/mL)		Cytotoxicity CC ₅₀ (mg/mL)
	Free	Bound	
WCC	17.92 ± 1.88 ^b	12.31 ± 0.83 ^b	>180
W15B	4.58 ± 0.39 ^d	1.39 ± 0.18 ^d	>180
BCC	27.18 ± 2.34 ^a	19.66 ± 1.02 ^a	>180
B15B	6.05 ± 0.18 ^c	2.11 ± 0.26 ^c	>180

Data expressed as mg /mL sample, DW; *n* = 3, values with different letter in each column are significantly different (*p* < 0.05)

WCC- wholemeal wheat cookie control, W15B- wholemeal wheat + 15 % blackcurrant powder, BCC- wholemeal barley cookie control, B15B- wholemeal barley + 15 % blackcurrant powder

10.3.6. Regulation of IL-1B, IL-6, NF-kB and NUCB/Nesfatin 1 genes expression in HepG2

Inflammation is one of the key biological processes activating cytokines genes in the development of all chronic diseases and autoimmune disorders (Choi et al. 2015). Interleukin-1 β (IL-1 β) and interleukin-6 (IL-6) are two prominent inflammatory cytokine genes which are thought to regulate inflammation. Up-regulation of cytokine genes related to chronic inflammations have been shown to be linked to the activation of nuclear transcription factor kappa B (NF-kB) signalling (Smolders et al. 2018). Controlling the regulation of those genes expression activates the immune response and enhances immunity.

The biological effects of phenolic extracts were investigated in HepG2 cells and assessed by RT-qPCR to determine the changes in three individual inflammation related genes regulation. Seeded cells were treated with 10 mg/mL phenolic extracts for 12 h. The regulation of cytokine genes expression as well as NF-kB transcription factor were shown down-regulated of all treatment (control vs tested samples), as shown in Figure-10.2. According to our results, antioxidant-DF extracts from blackcurrant rich samples (W15B and B15B) significantly ($p < 0.05$) suppressed IL-1 β and IL-6 genes expression about three to four-fold as well as inflammatory transcription factor NF-kB about two-fold, relative to samples from WCC and BCC respectively. These results followed the observations recorded for the higher TPC, chemical and cellular antioxidant capacity of W15B and B15B in comparison with WCC and BCC. This indicates that the potent biological activity is based on higher phenolic antioxidant, which is agreement with other previous studies (Kim et al. 2016; Strugała et al. 2016). Previous research has shown that bioactive phenolics interact with transcription factors and modulate activation of some signalling pathway and regulate genes involved in inflammatory mediated process (Vendrame & Klimis-Zacas 2015), this was reflected in our investigation.

Nucleobindin-2/nesfatin-1 (NUCB/Nesfatin-1) has been well documented as a hypothalamic satiety factor that controls appetite (Gao et al. 2016). Cumulative evidence has suggested that the up-regulation of NUCB plays an important role in controlling energy balance, reducing body weight and increasing satiety (Oh et al. 2006; García-Galiano et al. 2012). We observed a potential impact of

antioxidant-DF extracts on the regulation of NUCB/Nesfatin-1 gene expression at HepG2 cell model. The relative mRNA expression of W15B and B15B were markedly higher (about two to three-fold) in comparison with WCC and BCC. A bioactive-rich diet could increase the NUCB/Nesfatin-1 gene expression in human and mouse model (Shimizu et al. 2009; De Candia et al. 2017), however, the mechanisms of NUCB/Nesfatin-1 at cellular level remains unclear.

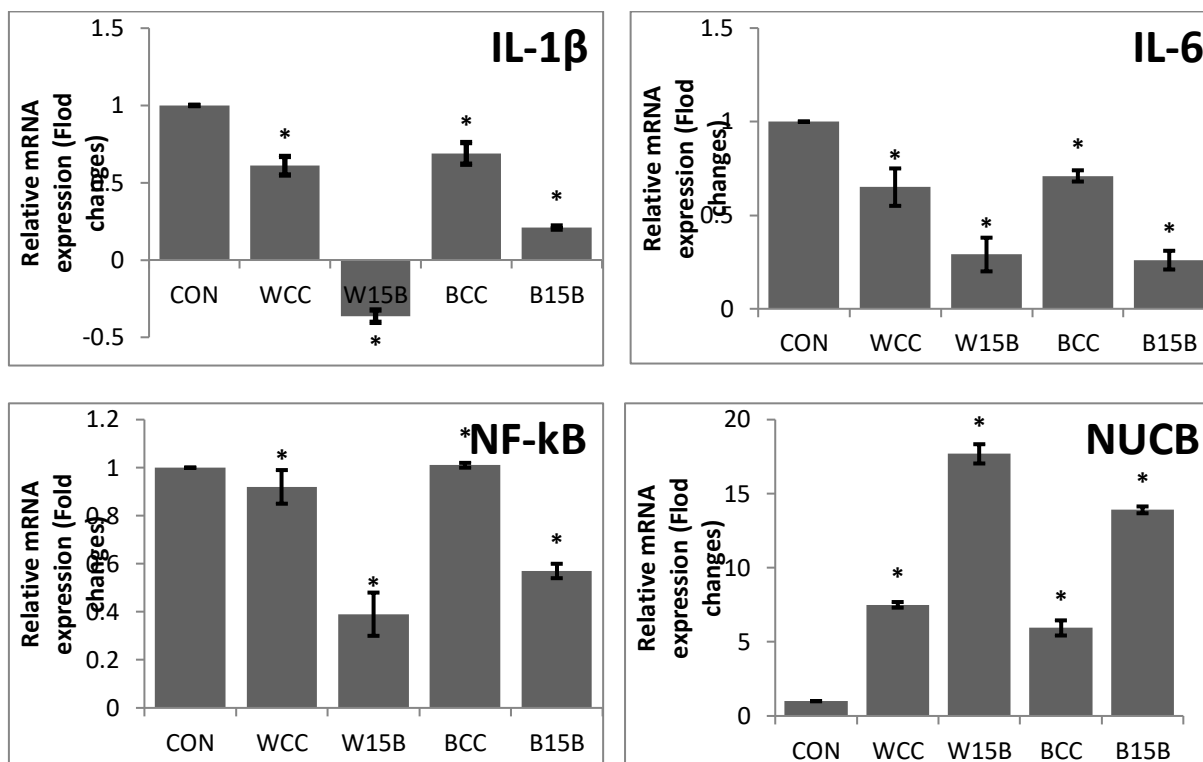


Figure 10.2. Relative mRNA expression (Fold change) of IL-1 β , IL-6, NF-kB and NUCB after treated with antioxidant-DF phenolic extracts of WCC, W15B, BCC and B15B (10 mg/mL) for 12 hours.

10.4. Conclusion

In summary, phenolic extracts of antioxidant-DF served to enhance bioactive phenolics, cellular antioxidant capacity, whilst inhibiting cancer proliferation and inducing the down-regulation of cytokine genes expression. Furthermore, the antioxidant-DF phenolic extracts potentially activated up-regulation of appetite regulator (NUCB/Nesfatin-1) gene.

While numerous studies have attempted to determine the bioactive elements of whole fruits and cereals and their biological activity, very few studies have investigated the undigested complex matrix of food components which are formed during the digestion of food, and to illustrate their potential health benefits. Our study demonstrated for the first time that the antioxidant-DF

components present after a simulated *in-vitro* digestion process act as potential sources of a wide range of bioactive molecules. This bioactive functionality of digested samples was linked to a role at cellular level, which could be the alternative perspective as a functional food to protect human health. The significant effect exhibited by blackcurrant rich material suggests that the antioxidant DF fractions of blackcurrant can persist the digestion process and be potent functional components.

Chapter 11

Antioxidant dietary fibre of astaxanthin-rich amicroalgae *Haematococcus pluvialis* incorporated wholemeal cereal cookie: potent bioactivity against HepG2 human cancer cell model

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To be submitted "Marine Drugs" Journal

Abstract

Astaxanthin incorporated antioxidant dietary fibre (antioxidant-DF) exhibited significantly higher radical scavenging capacity and genetic modulation on HepG2 cell model, which is the inverse relationship with many chronic diseases such as cancer. Radical scavenging capacity of phenolic extracts of antioxidant-DF was assessed chemically (ORAC) and at cellular biological system (CAA). The higher ORAC and CAA value indicated that astaxanthin incorporated antioxidant-DF (W15A and B15A) are strong antioxidants, which was in accordance with the higher phenolic content when compared with WCC and BCC. The major phenolic acids of the extracts were assessed by RP-HPLC, where ferulic acid, p-hydroxy benzoic acid and cinnamic acid demonstrated most dominant phenolic compounds, which exhibited the potential in the up-regulation of the anorexigenic NUCB-2/nesfatin gene expression. Down-regulation of the inflammatory cytokines (IL-1 β and IL-6) and inflammation activator (NF-kB) indicates a broad influence on inflammatory responses and enhancing immunity.

11.1. Introduction

Cancer is the leading cause of death globally, where diet has been considered pivotal in the aetiology and prevents most cancer (Bamia 2018). As the underlying molecular mechanisms of carcinogenesis have been revealed widely, the intensified link of specific dietary components have attracted much research interest. Therefore, naturally occurring bioactive molecules particularly polyphenols are the current subject of cancer research, due to their preventive potential in neurodegeneration, again, liquid malignancies and all form of cancers (Hasima & Ozpolat 2014; Lewandowska et al. 2016).

The term antioxidant dietary fibre (antioxidant-DF) is defined by “a dietary fibre concentrate containing significant amounts of natural antioxidants associated with non-digestible compounds” (Quirós-Sauceda et al. 2014). Complex matrix of food components particularly cell wall (nonstarch polysaccharides such as cellulose, hemicellulose) of whole-grains and vegetables are highly resistant or inaccessible to enzymatic digestion due to the densely packed physicochemical properties and/or not adequately disrupted during processing. Despite relatively little data are available, it becomes increasingly obvious that these antioxidant compounds along dietary fibre might have potential role to prevent radical induced carcinogenesis.

Whole grains are important source of nutrients, are also rich source of biologically active compounds predominantly dietary fibre (almost double than vegetables), vitamins, minerals and phytochemicals including antioxidants phenolic compounds which is in account of 15 % of total grain weight (FARDET 2013). Mounting evidence have hypothesized that the most food bioactive compounds have potential anticancer properties which is mainly attributed to the phenolic compounds, but they may be present in free and/or bound forms found in the cell walls (Salawu et al. 2014). For example, ferulic acids may be bound to the hemicellulose fibre (Acosta-Estrada et al. 2014).

Microalgae such as *Haematococcus pluvialis* are untapped natural resources that have high aggregated bioactive compounds such as carotenoids (Ota et al. 2018). *H. pluvialis* microalgae is a rich sources of antioxidant astaxanthin, which exerts several fold higher radical scavenging capacity than β -carotene and vitamin E, have been demonstrated in several *in-vitro* and animal studies (Ni et al. 2015). However, earlier studies on marine biology, a few toxicological trials on a limited number of marine algae have shown the toxicity from the ingestion, rather wide varieties are unidentified. The toxicity of microalgae might be species specific, but needed for an extensive investigation on human cell model. Several studies have hypothesized that less digestibility of biologically important microalgae is the cell wall integrity, which may not be sufficiently disrupted during processing and/or hydrolysed by human gastrointestinal enzymes (Kousoulaki et al. 2015). If the bound bioactive compounds released and absorbed, could be potential for human health and diseases. The esterases present in the human intestinal tract that may active to such dietary bioactive compounds (Borges et

al. 2013). Colonic digestion of such compounds may have released and digested, possess potential health benefits (Adom & Liu 2002).

Therefore, the present study investigated the potential of antioxidant dietary fibre of the astaxanthin-rich microalgae incorporated wholemeal flour cookie against cellular biological system.

11.2. Materials and methods

11.2.1. Chemicals and reagents

The chemicals and reagents were used for this study was described in section 3.1.

11.2.2. Total dietary fibre (TDF) isolation from cookies

Total dietary fibre (TDF) isolation from model food was carried out as described in section 3.12.

11.2.3. Free and bound phenolics extraction from TDF

The free and bound phenolics extraction from TDF were carried out as described in section were described in section 3.8.1 and 3.8.2.

11.2.4. Total phenolic content determination

The total phenolic content of free and bound phenolics extract were determined as described in section 3.8.

11.2.5. Chemical antioxidant analysis by ORAC

Described in section 3.9.2.

11.2.6. Major phenolic acids composition determined by RP-HPLC

Described in section 3.10.

11.2.7. Cell culture and culture condition

Described in section 3.18.

11.2.8. Cellular antioxidant analysis (CAA) of antioxidant-DF extracts

Described in section 3.19.

11.2.9. Cytotoxicity and anti-proliferative activity

Described in section 3.20.

11.2.10. Quantitative real-time PCR and regulation of gene expression

Described in section 3.21.

10.2.11. Statistical analysis

Described in section 3.24.

11.3. Result and Discussion

11.3.1. Total phenolic content (TPC) of antioxidant-DF

Phenolic compounds are the major contributor to the various biological functions, particularly antioxidant properties. The total phenolic content of the extract differed significantly ($p < 0.05$) among wholemeal wheat and barley fibre (WCC and BCC), and 15 % astaxanthin incorporated wholemeal wheat and barley fibre (W15A and B15A), results are presented in Table 11.1. W15A presented the highest phenolic content (721.24 mg GAE/100 g sample, DW) which is about 30 % higher when compared with WCC (500.50 mg GAE/100 g sample, DW) followed by B15A (625.28 mg GAE/100 g sample, DW) BCC which is about 33 % higher when compared with BCC (412.99 mg GAE/100 g sample, DW). These could be due to the higher phenolics of astaxanthin-rich microalgae *H. pluvialis* (Goiris et al. 2012). The bound phenolic fraction exerted higher TPC in all treatment fractions, which is agreement with previous studies (Sosulski et al. 1982; Acosta-Estrada et al. 2014). Alkaline digestion significantly improved the release of bound phenolics of wholemeal cereals prior to extraction, which might have the higher surface contact, breakdown cell wall of the complex food matrices and lower rate of phenolics loss (Krygier et al. 1982). Importantly, the enhanced phenolics of antioxidant-DF that is higher than some cereals and fruits (Shahidi & Yeo 2016), which could be the potential ingredients for the functional food industry.

Table 11.1. Free, bound and total phenolic content of antioxidant-DF extract

Sample	Free phenolics	Bound phenolics	Total phenolics
WCC	191.88 ± 6.86 ^c	308.62 ± 3.10 ^c	500.50 ± 9.96 ^c
W15A	319.15 ± 10.01 ^a	402.09 ± 13.03 ^a	721.24 ± 23.04 ^a
BCC	148.47 ± 6.10 ^d	264.52 ± 5.99 ^d	412.99 ± 12.09 ^d
B15A	280.14 ± 7.42 ^b	345.14 ± 10.72 ^b	625.28 ± 18.14 ^b

Triplicate data expressed as MEAN mg GAE/100 g sample, DW; values with different letter as superscripts in each column indicates significantly different ($p < 0.05$)

11.3.2. Chemical antioxidant analysis of antioxidant-DF

Phenolic compounds are powerful antioxidants, which have reportedly been considered as scavengers to prevent oxidative damage. The antioxidant potential of the phenolic fractions of antioxidant-DF were assessed by oxygen radical absorbance capacity (ORAC) assay, results are presented in Table 11.2. The antioxidant-DF phenolic fractions were overall effective scavengers, but the significantly ($p < 0.05$) enhanced radical absorbance capacity was demonstrated astaxanthin incorporated fraction W15A (189.55 $\mu\text{mol TE/g}$ sample, DW) followed by B15A (106.52 $\mu\text{mol TE/g}$ sample, DW) when compared with WCC (173.79 $\mu\text{mol TE/g}$ sample, DW) and BCC (85.74 $\mu\text{mol TE/g}$ sample, DW) respectively. Inclusion of astaxanthin-rich microalgae *H. pluvialis* might enhance interaction and chelating potential in biochemical ORAC assay, which is widely reported by previous studies (Régnier et al. 2015; Focsan et al. 2017). Surprisingly, wholemeal wheat antioxidant-DF fraction (WCC) showed significantly higher scavenging capacity when compared with wholemeal barley fraction (BCC), indicating that the potential bioactive molecules released during extraction that possessed potent antioxidant ability (Krygier et al. 1982; Shahidi & Yeo 2016). These results were positively associated with the total phenolic contents. Antioxidant ability mostly attributed to the chemical structure, redox properties of bioactive molecules and potent individual molecule, could be available in the antioxidant-DF extracts demonstrated significant biological effects.

Table 11.2. Total antioxidant capacity (ORAC values) of antioxidant-DF fractions

Sample	Free	Bound	Total
WCC	73.11 \pm 4.46 ^a	100.68 \pm 3.27 ^a	173.79 \pm 7.73 ^b
W15A	78.08 \pm 3.93 ^a	111.47 \pm 6.11 ^a	189.55 \pm 10.04 ^a
BCC	28.42 \pm 2.67 ^b	57.32 \pm 3.49 ^c	85.74 \pm 6.16 ^d
B15A	33.13 \pm 2.77 ^b	73.39 \pm 4.28 ^b	106.52 \pm 7.05 ^c

Triplicate data expressed as MEAN $\mu\text{mol TE/g}$ sample, DW), values with different letter as superscripts in each column indicates significantly different ($p < 0.05$)

11.3.3. Phenolic profile of antioxidant-DF

Identification of phenolic compounds are of great interest due to their diverse biological role in human body. The extracts of antioxidant-DF exhibited wide range of phenolic compounds, detected by RP-HPLC, analytical results are presented in Table 11.3. Total eight phenolic acids (p-hydroxy benzoic acid, vanilic acid, syringic acid, caffeic acid, *p*-coumaric acid, ferulic acid, cinnamic acid and salicylic acid) were identified as major phenolic acids in the extracts. The data showed that bound phenolic fraction exhibited higher concentration of identified phenolic acids. p-hydroxybenzoic acid, ferulic acid and cinnamic acid were the most abundant phenolic acid, which is in agreement with previous studies (Safafar et al. 2015; Shamloo et al. 2017). Cinnamic acid and salicylic acid were identified in the astaxanthin-rich microalgae *H. pluvialis* incorporated antioxidant-DF extracts W15A and B15A (Safafar et al. 2015). Importantly, the identified and dominant phenolic acids of the extraction have been extensively reviewed, exerted higher bioavailability and potential to systemic and gut health (Mnafgui et al. 2015; Roleira et al. 2015).

Table 11.3. Quantification of major phenolic acids, triplicate data expressed as mg phenolic acid /100 g sample, dry weight

Sample	Fraction	p-hydroxy benzoic acid	vanilic acid	syringic acid	caffeic acid	p-coumaric acid	ferulic acid	cinnamic acid	salicylic acid
WCC	Free	7.82 ± 0.09	8.94 ± 0.48	11.79 ± 0.71	13.01 ± 0.47	9.67 ± 0.81	9.30 ± 0.75	nd	nd
WCC	Bound	15.33 ± 0.54	6.77 ± 0.52	20.98 ± 0.12	10.01 ± 0.74	10.73 ± 0.06	6.50 ± 0.06	nd	nd
WCC	Total	23.15 ± 0.63	15.72 ± 1.00	32.78 ± 0.83	23.03 ± 1.21	20.41 ± 0.87	15.80 ± 0.81	nd	nd
W15A	Free	17.61 ± 0.38	6.21 ± 0.13	11.29 ± 0.16	7.88 ± 0.39	11.42 ± 0.17	11.99 ± 0.67	16.24 ± 1.01	09.54 ± 0.05
W15A	Bound	19.86 ± 0.76	7.29 ± 0.21	16.97 ± 1.03	8.59 ± 0.25	13.71 ± 0.42	16.68 ± 0.53	20.06 ± 1.22	14.65 ± 0.46
W15A	Total	37.47 ± 1.14	13.50 ± 0.34	28.26 ± 1.19	16.47 ± 0.64	25.13 ± 0.59	28.67 ± 1.20	36.30 ± 2.23	24.19 ± 0.51
BCC	Free	10.81 ± 0.66	2.51 ± 0.02	nd	12.75 ± 0.55	9.94 ± 0.41	16.07 ± 1.58	nd	nd
BCC	Bound	21.52 ± 0.64	2.61 ± 0.03	nd	21.56 ± 0.54	10.14 ± 0.09	19.12 ± 0.68	nd	nd
BCC	Total	32.34 ± 1.30	5.13 ± 0.05	nd	34.32 ± 1.09	20.09 ± 0.50	35.20 ± 2.26	nd	nd
B15A	Free	11.13 ± 0.68	2.89 ± 0.13	nd	13.87 ± 0.79	11.11 ± 0.64	17.11 ± 1.12	18.67 ± 1.00	11.33 ± 0.45
B15A	Bound	14.38 ± 0.29	2.52 ± 0.02	nd	23.98 ± 1.23	14.67 ± 1.01	21.01 ± 1.30	22.31 ± 1.49	15.31 ± 1.05
B15A	Total	25.51 ± 0.97	5.41 ± 0.15	nd	37.85 ± 2.02	25.78 ± 1.65	38.12 ± 2.42	40.98 ± 2.49	26.64 ± 1.50

11.3.4. Cytotoxicity and anti-proliferative effects of antioxidant-DF

Despite microalgae are the most important source of potent biologically active compounds, some species of dinoflagelates. Hence, toxicological evaluation of *H. pluvialis* microalgae incorporated antioxidant-DF fractions established its safe consumption. HepG2 cells were treated with different concentration of antioxidant-DF phenolic fractions, results are expressed as CC_{50} and presented in Table 11.4. An important evaluation that there were no considerable toxic effects showed at the applied dose against HepG2 cell model, similar result demonstrated previously (Régnier et al. 2015). Uncontrollable cell growth and/or proliferation is a hallmark of the pathogenesis of all form of cancer. Hence, inhibition of cell proliferation is a major therapeutic target of cancer disease. Anti-proliferation activity of antioxidant-DF fractions were evaluated with the different concentration of tested samples against human cancer cell line, HepG2. The phenolic extracts of all tested samples were significantly exhibited anti-proliferative in dose and time-dependent manner (1 to 10 mg/mL, 24 h to 72 h), assessed against CM control. The results are presented in Table 11.4 as median effective concentration (EC_{50}), where, lower value indicates higher anti-proliferative effects of the tested compounds. Bound phenolic extracts were demonstrated higher effects against HepG2 cell growth inhibition than free phenolic extracts. However, significantly ($p < 0.05$) higher inhibitory effects (lower EC_{50} value) were demonstrated by the W15A (2.14 mg/ml sample, DW) followed by B15A (4.59 mg/ml sample, DW), WCC (12.31 mg/ml sample, DW) and BCC (19.66 mg/ml sample, DW) respectively. Similarly, free phenolic extracts of W15A and B15A were also demonstrated significantly higher inhibitory effects in cell proliferation, which could be synergistic and/or individual effects of phenolic compounds (Bilbao et al. 2016). The significant anti-proliferative effects of antioxidant-DF could be due to the higher affinity of the molecules and their intracellular activity suppress proliferative signalling and cell growth (Nagaraj et al. 2012; Zhu et al. 2015). Importantly, *H. pluvialis* incorporated antioxidant-DF W15A and B15A contained cinnamic and salicylic acid which have potent biological properties (Nagaraj et al. 2012; Mnafigui et al. 2015).

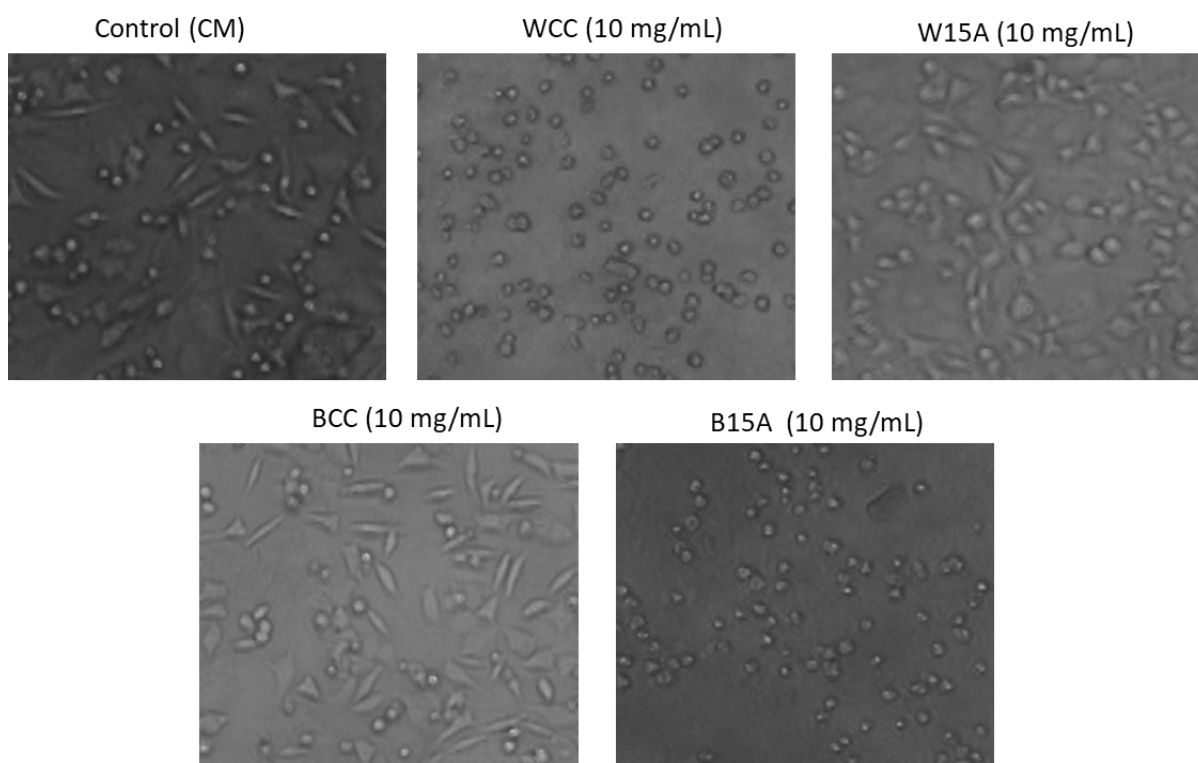


Figure 11.1. Anti-proliferative activity on HepG2 cell line (under microscope observation). Cells treated with antioxidant-DF for 72 h

Table 11.4: Anti-proliferative activity and cytotoxicity of antioxidant-DF on HepG2 cell line

Sample	Anti-proliferation EC ₅₀ (mg/mL)		Cytotoxicity CC ₅₀ (mg/mL)
	Free	Bound	
WCC	17.92 ± 1.88 ^b	12.31 ± 0.83 ^b	>180
W15A	6.07 ± 0.85 ^d	2.14 ± 0.31 ^d	>180
BCC	27.18 ± 2.34 ^a	19.66 ± 1.02 ^a	>180
B15A	8.41 ± 0.79 ^c	4.59 ± 0.73 ^c	>180

Triplicate data expressed as mg /mL sample, DW; values with different letter in each column are significantly different ($p < 0.05$)

11.3.5. Cellular antioxidant analysis (CAA) of antioxidant-DF

Antioxidant capacity in living cells is relevant to the complex biological system including molecular bioavailability. The CAA technique exhibits physiological antioxidation that overcomes the limitation of chemical analysis techniques. Cellular antioxidant capacity of antioxidant-DF was investigated against HepG2 cell model, results are presented in Table 11.5. In the system, DCFH oxidises to DCF by the exogenous peroxy radicals ABAP that proportionately attributes to the fluorescence which is reduced by the antioxidant compounds. The antioxidant-DF phenolic fractions were overall effective scavengers, indicated that the extracts had ability to be absorbed by the cells in dose-response manner. The higher CAA value indicates better antioxidation. The significantly ($p < 0.05$) higher

radical scavenging ability was demonstrated by the extracts from W15A (476.46 $\mu\text{mol QE/ 100 g}$ sample, DW) and B15A (416.55 $\mu\text{mol QE/ 100 g}$ sample, DW) when compared with WCC (396.44 $\mu\text{mol QE/ 100 g}$ sample, DW) and BCC (161.87 $\mu\text{mol QE/ 100 g}$ sample, DW) respectively. However, “no PBS wash” protocol exhibited higher CAA values than “PBS wash” in all treatment samples. This could be due to the weak interaction of the compounds and cell surface, compounds solubility, permeability and their potential intracellular activity (Geng et al. 2016). However, the CAA values are partly ascribed to the ORAC value, but the stronger antioxidant ability (several folds higher) of the extracts exhibited in the cell model. This observation implies the redox potential of the compounds in the complex biological system than direct quenching.

Table 11.5. Cellular antioxidant activity (CAA) of antioxidant-DF

Sample	Free		Bound		Total	
	No PBS wash	PBS wash	No PBS wash	PBS wash	No PBS wash	PBS wash
WCC	302.74 \pm 11.49 ^c	152.90 \pm 8.43 ^c	435.94 \pm 10.44 ^c	243.54 \pm 8.99 ^c	738.68 \pm 21.93 ^c	396.44 \pm 17.42 ^c
W15A	342.81 \pm 11.88 ^a	198.44 \pm 7.01 ^a	509.13 \pm 10.21 ^a	278.02 \pm 6.68 ^a	851.94 \pm 22.09 ^a	476.46 \pm 13.69 ^a
BCC	127.38 \pm 6.92 ^d	60.08 \pm 3.91 ^d	226.98 \pm 7.20 ^d	101.78 \pm 2.73 ^d	354.37 \pm 14.12 ^d	161.87 \pm 6.64 ^d
B15A	289.16 \pm 9.21 ^b	159.91 \pm 5.54 ^b	484.11 \pm 9.98 ^b	259.64 \pm 8.55 ^b	773.27 \pm 19.19 ^b	416.55 \pm 14.09 ^b

Triplicate data expressed as $\mu\text{mol QE/ 100 g}$ sample, DW); $n = 3$, values with different letter in each column are significantly different ($p < 0.05$)

11.3.6. Regulation of IL-1 β , IL-6, NF-kB and NUCB-2/Nesfatin genes expression in

HepG2

Chronic inflammation is a well-recognised feature of cancerous conditions. Up-regulation of pro-inflammatory cytokines such as interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and nuclear transcription factor (NF-kB) are the key activator of inflammation. Our study investigated the inflammation related genes expression on HepG2 cell model, assessed by RT-qPCR. All antioxidant-DF extracts exerted significantly down-regulation of IL-1 β , IL-6 and transcription factor NF-kB expression when compared with untreated control, results are presented in Figure 11.2. However, astaxanthin incorporated phenolic extracts W15A and B15A demonstrated higher activity in order to suppress the regulation of IL-1 β (about four-fold) and IL-6 (about two-fold), and transcription factor NF-kB (about quarter-fold) when compared with WCC and BCC respectively. These results in accordance with the higher phenolic content and their biological activity as demonstrated in ORAC and CAA assay, supported by

previous studies (Bae et al. 2015). Importantly, polyphenolic antioxidants are potent inhibitors of oxidative stress, chronic inflammation and associated carcinogenesis, investigated in mice model (Bae et al. 2015). Previously reported, astaxanthin suppressed transcription factor NF- κ B signalling and inflammatory cytokine in high-fat diet fed mice (Huang et al. 2015), as reflected in our study.

Nucleobindin-2/nesfatin (NUCB/Nesfatin) is a well-studied anorexigenic hormone, involved in the regulation of gastrointestinal functions, increased satiety and energy homeostasis (Stengel 2015). Our study investigated the impact of antioxidant-DF on the regulation of NUCB-2/nesfatin in HepG2 cell model. All treated samples were greatly up-regulated the NUCB/nesfatin genes when compared with non-treated control, results are presented in Figure 11.2. Astaxanthin incorporated extracts demonstrated markedly higher (about eleven-fold higher compared to control and about two-fold higher when compared with WCC and BCC) effects on NUCB-2/nesfatin up-regulation. This suggests that compounds of phenolic extract that are well absorbed and responsible for the NUCB-2/nesfatin stimulation effect. Previous studies supported that the bioactive compounds rich diet significantly increased NUCB-2/nesfatin, mediating satiety effects in mice and human (De Candia et al. 2017; Rodriguez et al. 2017). Acute modulation of NUCB-2/nesfatin gene expression by polyphenols has also shown on *in-vitro* model (García-Galiano et al. 2012; Stengel 2015). Although antioxidant-DF extract is composed of several different compounds responsible for this direct stimulation, further study is needed to understand particularly, the astaxanthin effects on genetic regulation in more details.

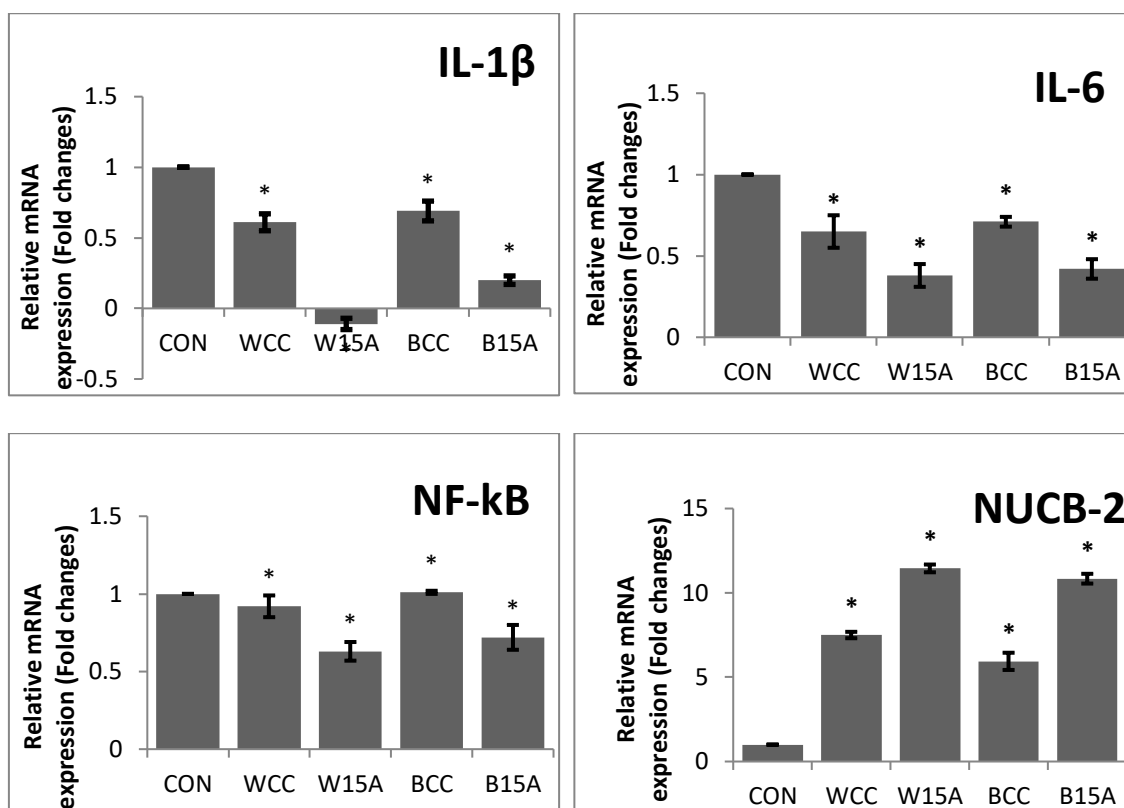


Figure 11.2. Relative mRNA expression (Fold change) of IL-1 β , IL-6, NF-kB and NUCB-2 after treated with antioxidant-DF phenolic extracts of WCC, W15A, BCC and B15A (10 mg/mL) for 12 hours. Asterisks (*) indicated statistically significant ($p < 0.05$) between treated and non-treated control (CON) samples.

11.4. Conclusion

Astaxanthin incorporated phenolic extract was more effective in chemical and cellular antioxidant activity than was only wholemeal cereals antioxidant-DF in HepG2 cell model. The study suggest that phenolic extracts of antioxidant-DF could reduce the chronic inflammation, oxidative stress and associated pathological condition particularly cancer, as shown by the down-regulation of pro-inflammatory cytokine and inflammation activation factor. Moreover, up-regulation of NUCB-2/nesfatin could able to improve satiety and metabolic homeostasis.

Chapter 12

The microbial metabolites, short-chain fatty acids promote amyloid- β aggregation in cell model of Alzheimer's disease

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Abstract

Human gut microbes have emerged as a significant influence on nutrient metabolism, gastrointestinal physiology, immune function and brain-gut regulation. Indeed, major microbially produced metabolites are short-chain fatty acids (SCFAs). The status of gut microbes have been investigated widely in relation to peripheral immunity and blood-brain barrier integrity. The current study used cell model system to demonstrate a critical role of SCFAs in amyloid-beta ($A\beta$) aggregation. The Alzheimer's disease (AD) linked E22G-mCherry Hek 293 cell model study demonstrated that *in-vitro* bacterial fermenta promoted $A\beta$ aggregation rate at higher SCFAs concentration (0.5 mM) when compared with the control (medium only). The lower SCFAs concentration (0.1 mM) had no significant impact on direct inhibition/progression of $A\beta$ aggregation, as demonstrated by flow cytometry and image analysis. Hence, the results reveal that higher concentration of bacterial metabolites SCFAs represent a link to the risk of neurodegeneration in the AD cell model, which closely mimics the progression of human AD-like pathogenesis.

12.1. Introduction

Amyloid disorders are the hallmark of numerous neurodegenerative diseases, particularly Alzheimer's disease (AD). Amyloid-beta ($A\beta$) aggregation and/or fibril/plaques formation are the major pathophysiological features of AD (Paulson et al. 2008; Sadigh-Eteghad et al. 2015). Metabolic dysfunction of amyloid precursor protein (APP) leads to the increased production and accumulation of $A\beta$ as fibril and/or plaques, which are the crucial events of neuro-toxicity, neuro-inflammation and

subsequently cell death, neuronal loss and neurodegeneration (Brettschneider et al. 2015; Heneka et al. 2015). Over the past decade, studies into gut-brain axis explored how the gut influences neurological conditions. Several mouse model studies have reported gut-microbiota produced metabolites impaired blood-brain barrier function (Braniste et al. 2014), neurotropic factor (Bercik et al. 2011), affects anxiety and cognition (Clarke et al. 2013). However, no particular study has demonstrated the direct link between microbes, metabolites and amyloidosis of AD.

Diet drives gut microbes composition, making a connection between gut and different physiological states via microbial fermented metabolites. Dietary fibres which escape upper gastrointestinal digestion, are fermented by the gut microbes, produce as major metabolites, short-chain fatty acids (SCFAs) such as acetate, propionate and butyrate (Macfarlane & Macfarlane 2012). Pathophysiologic evidence suggested that the effects of microbial metabolites SCFAs extend beyond the gut. SCFAs are major energy source of enterocytes and act as signalling molecules to affect peripheral organs via activation of hormone and nervous systems. A recent study revealed a link between SCFAs and microglia alteration, as SCFA can cross the blood-brain barrier (BBR) (Frost et al. 2014; Erny et al. 2015). However, the direct link between microbial metabolites and A β aggregation of AD is not fully explored.

The current study investigated whether the fermented metabolites can inhibit the progression of A β aggregation in an AD cell model. We analysed A β aggregation rate (image and flow cytometric analysis) of AD linked E22G-mCherry Hek 293 cells, treated with different concentrations of fermented metabolites and compared with the cells in culture media under the same *in-vitro* culture condition.

12.2. Materials and methods

12.2.1. Samples

The present study used the fermented metabolites as test samples. The sample preparation was described in section 3.16.

12.2.2. Cell culture and culture condition

Described in section 3.18

12.2.3. Determination of A β -aggregation (image analysis)

Described in section 3.22.

12.2.4. Determination of A β -aggregation (flow cytometry analysis)

Described in section 3.23.

12.2.5. Statistical analysis

Described in section 3.24

12.3. Result and discussion

E22G is a pathogenic mutation affecting the processing of amyloid precursor protein (APP), resulting in increased toxic A β aggregates of AD (Lashuel et al. 2003; Yoo et al. 2018). To examine the effect of bacterial metabolites on A β aggregation, we conducted image analysis and flow cytometry of fluorescently labelled AD-linked E22G-mCherry Hek 293 cells. Under the culture condition, A β aggregation appeared among control and treated cells during the 96 h incubation period. The magnitude of different fermenta effects on A β aggregations are presented in Figure 12.1-12.6 and Table 12.1.

The level of intracellular A β protein aggregation in E22G-mCherry Hek 293 cell model varied with 0.1 mM and 0.5 mM SCFAs concentration of the three different samples WCC, W15B and W15A. High magnification fluorescence microscopic images of A β demonstrated that there was a positive correlation between the A β aggregation level and SCFAs concentration of WCC, W15B and W15A samples. Importantly, no significant impact as observed in the size of the A β deposition in cells treated with 0.1 mM SCFAs when compared to the control cells treated with medium only. SCFAs act as a signalling molecule in cellular biological process (Koh et al. 2016). The higher concentration of these signalling molecules may induce excessive stimulation, inflammation and activate cells to initiate A β production (Vinolo et al. 2011).

E22G-mCherry Hek 293 single cell was identified and quantified using forward scatter flow

cytometry. The A β -aggregates spot count, histograms of A β -aggregates on the single cells and representative images of cells with or without A β -aggregates were taken from gate R1 and R2 and presented in figure 12.1-12.6 and table 12.1. The percentage of A β -aggregates was progressively increased in all treatment after 96 h incubation, demonstrating the activity of E22G mutation. The 0.5 mM SCFAs concentration of WCC, W15B and W15A fermenta significantly increased the A β -aggregates compared with control. Surprisingly, fermenta of blackcurrant incorporated fibre fraction exhibited trend to increased A β -aggregates compared with WCC and W15A samples, which might be due to different distribution of SCFAs or the higher concentration of particular SCFA available in the fermenta (Kleman & Strohl 1994; Wong et al. 2006). Cellular uptake and/or binding capacity of W15B fermented molecule to the cellular system may affect the A β -aggregates (Locasale & Cantley 2011; Tremaroli & Bäckhed 2012), which might not significantly available in WCC and W15A fermenta. A β -aggregations were varied in size in the cells treated with the different concentration of SCFAs fermenta. A β -aggregates appeared larger in cells treated with higher concentration SCFAs.

Although, numerous previous studies demonstrated the protective role of gut metabolites in neurodegeneration (Ho et al. 2016; Pistollato et al. 2016; Ho et al. 2018), this study demonstrated the opposite result which is the extending observation of recent investigation (Smith 2015; Lin et al. 2018). Recently, it was revealed that oral administration of SCFAs promotes neuroinflammation in germ-free mice, which is consistent with our current study (Sampson et al. 2016). Even though the diversity of microbial taxa significantly altered the abundance and profile of SCFAs, our study fermenta was only from the composition of four bacterial species (*Lactobacillus* and *Bifidobacterium*). Furthermore, the effect of total fermenta might be different to the effects of specific SCFA such as acetate, propionate and butyrate, which may have specific mechanism of action in cellular biological process which attenuates the A β - aggregates. However, widely studied microbial metabolites SCFAs promote neurodevelopment. There are likely underlying link/biological function which may accelerate the process of neurodegeneration (Minter et al. 2016). The ability of gut metabolites to enhance this critical cellular biological function may revealed the link between the gut and brain disorder like AD.

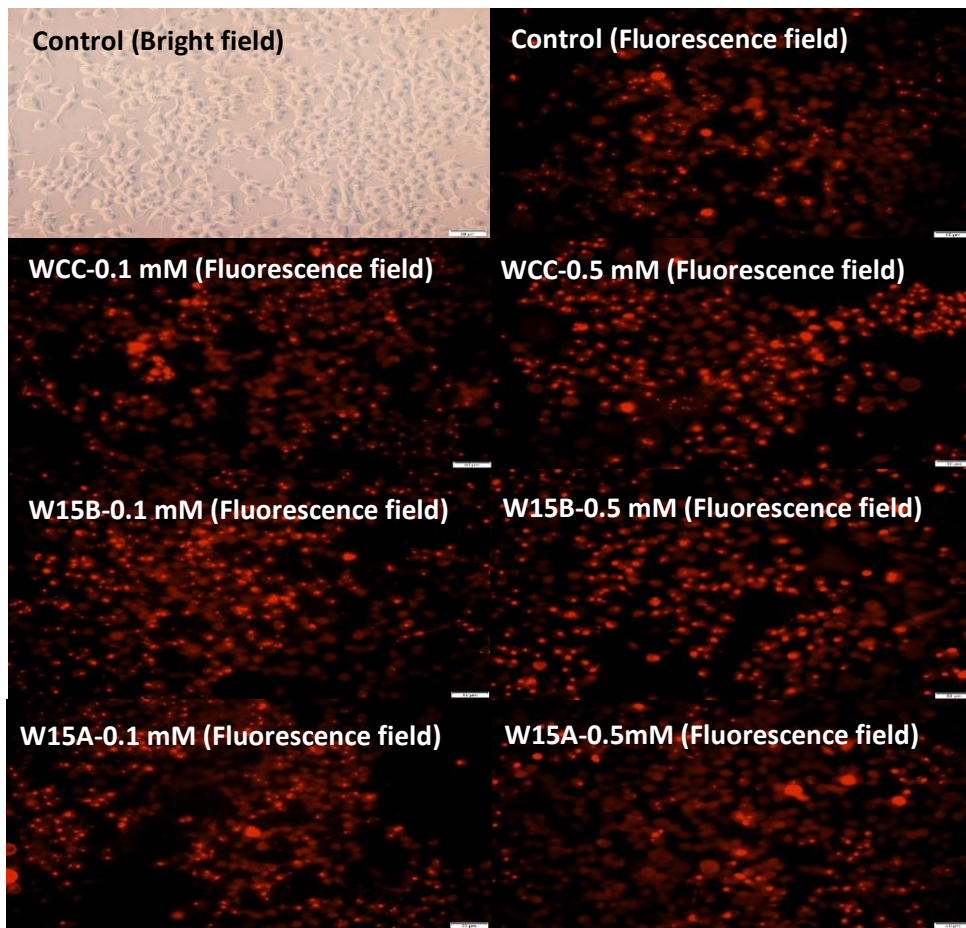


Figure 12.1. Images of different fermented samples (SCFAs concentration 0.1 mM and 0.5 mM) applied on the cell model.

Abbreviations: WCC- wholemeal wheat cookie control, W15B- wholemeal wheat + 15 % blackcurrant powder, W15A- wholemeal wheat + 15 % astaxanthin powder

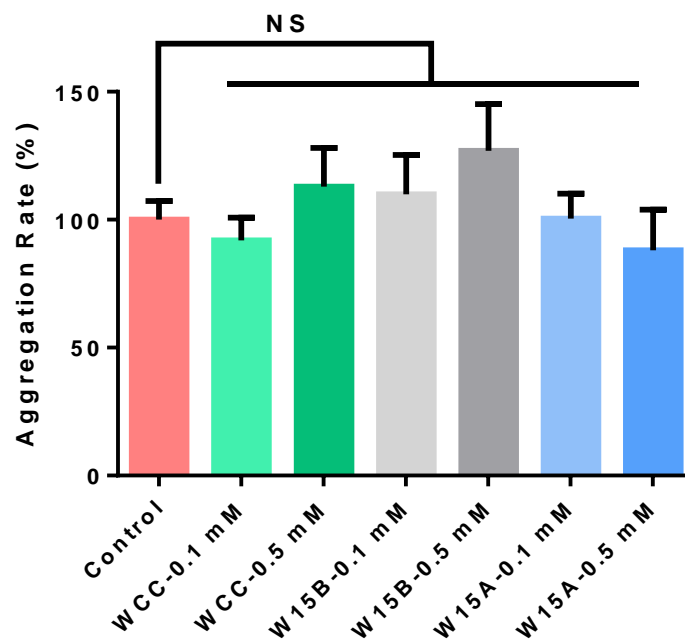


Figure 12.2. Aggregation rate of different fermented samples applied to the cell model. NS (not significant, $p < 0.05$)

Table 12.1. Analysis of the A β aggregates in the cell model using flow cytometry

Sample	R1 Gated (%)	R2 Gated (%)	Spot counts 0-10 (%)	Spot counts 10-20 (%)	Spot counts 20-30 (%)	Spot counts 30-40 (%)
Control	25.20	74.40	60.51	55.60	51.52	50.96
WCC	22.90	75.90	27.82	28.91	31.29	31.76
W15B	18.60	80.60	9.12	11.44	13.22	12.96
W15A	21.30	77.40	2.55	4.05	3.97	4.32

Anti-aggregation effect of fermented WCC, W15B and W15A fibre on the cell line (flow cytometry)

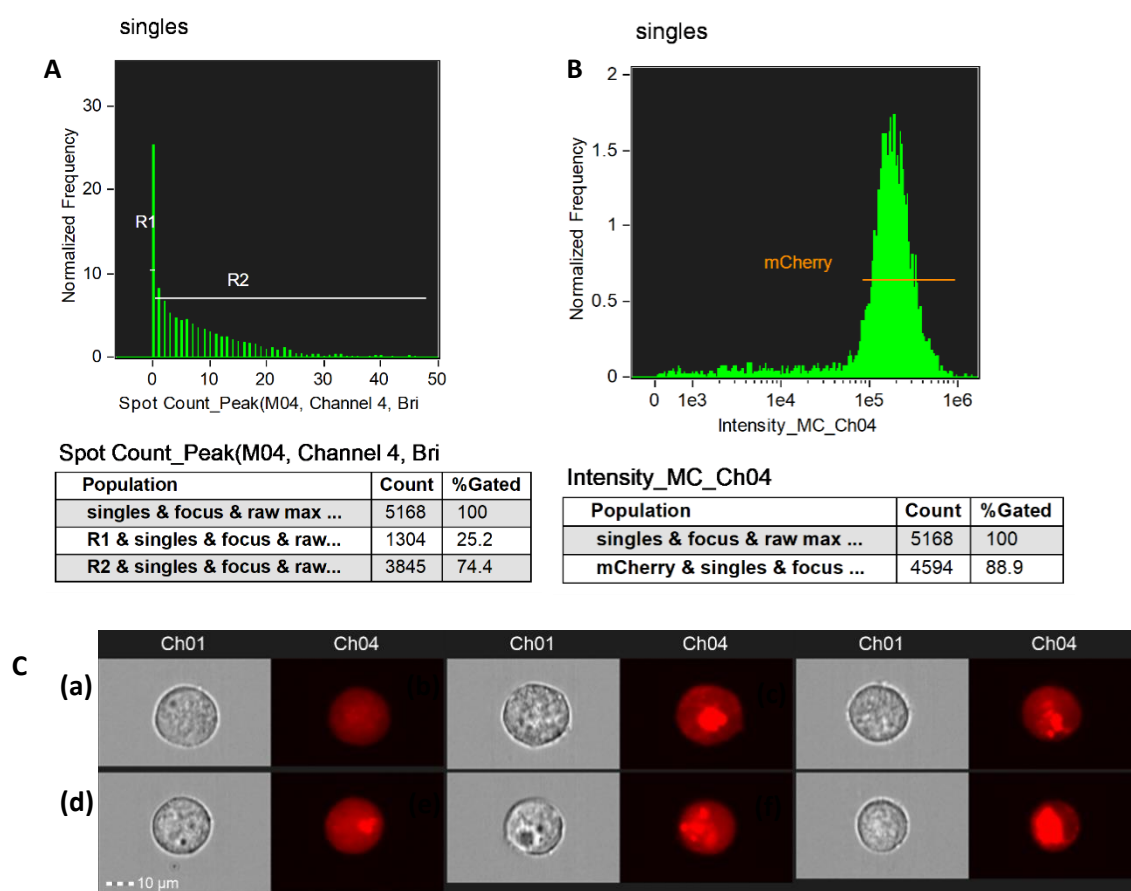


Figure 12.3. Image flow cytometry analysis of model control group. (A) Spot count analysis of A β aggregates. (B) Histograms of A β -aggregates on the single cells. (C) Representative images of cells with or without A β aggregates.

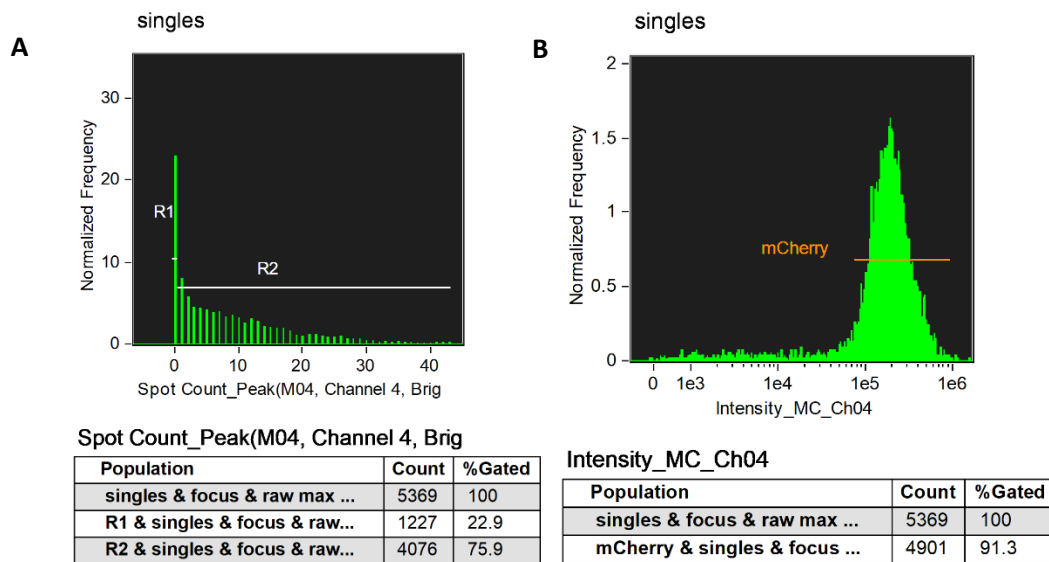


Figure 12.4. Image flow cytometry analysis of WCC (0.1 mM) group. (A) Spot count analysis of A β aggregates. (B) Histograms of A β -aggregates on the single cells.

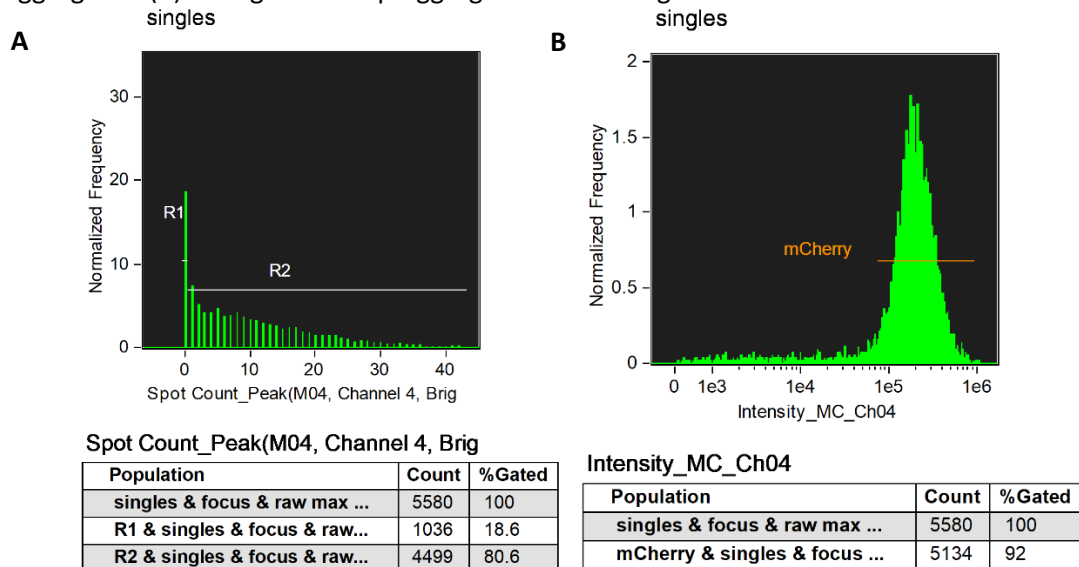


Figure 12.5. Flow cytometry analysis of W15B (0.1 mM) group. (A) Spot count analysis of A β aggregates. (B) Histograms of A β -aggregates on the single cells.

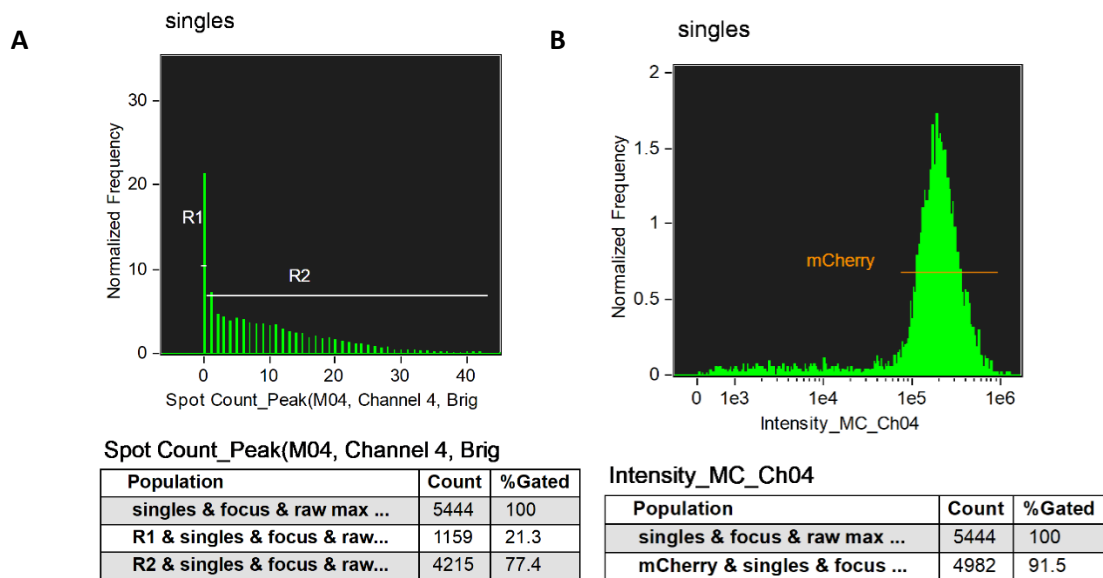


Figure 12.6. Image flow cytometry analysis of W15A (0.1 mM) group. (A) Spot count analysis of A β aggregates. (B) Histograms of A β -aggregates on the single cells.

12.4. Conclusion

Coupled with emerging research, this cell model study demonstrated the critical role of gut metabolites in the progression of A β -aggregation of AD. Besides the diverse functional role of SCFAs in a number of organs, understanding the concentration of metabolites/SCFAs and their cellular biological activity in protective health benefits are warranted.

Chapter 13

General discussion and conclusion

Numerous epidemiological studies have reported health benefits of bioactive food components (Liu 2004; Rodriguez-Mateos et al. 2014; Xiao & Hogger 2015). The results of the current research provide novel insights into possible mechanisms. In particular, this study aimed to investigate the effect of bioactive compound-rich wholemeal cereals (wheat, barley and oats) cookie made with fruits (blackcurrant) and microalgae *Haematococcus pluvialis* on glycaemic control, enhancement of growth of beneficial gut bacteria, production of bacterial metabolites, SCFAs, and their effects on the regulation of gut hormones in order to control appetite and satiety. While wholegrain cereals, berries and marine food microalgae consumption has been suggested, to bring potential to improve health and diseases (Kris-Etherton et al. 2002; Nile & Park 2014; Talero et al. 2015). The results revealed the beneficial relationship of dietary bioactive compounds in cellular anti-proliferation, antioxidation, pro-inflammatory cytokine regulation and appetite regulation and risk markers associated with causing neurological disorders and overall life span. A combination of dietary fruits and fibre-rich cereals may reap benefits on multiple levels, which require investigation of their nutritional attributes for the specific physiological effects. This study demonstrated the implication of bioactive components richness in a cookie may provide health benefits, specifically anti-obesity, anti-diabetic and anti-cancer effects *in-vitro*, chemical and in a cell model. This study also demonstrated the direct link of gut metabolites in progression of A β -aggregates in a cell model of AD, which could provide novel insight to accelerate our knowledge of amyloidogenic disorders.

Preparation and cooking can enhance the nutritional quality and quantity in food. In particular, they can improve the availability and stability of bioactive compounds, which exerts significant impact on digestibility and bioactivity (Foschia et al. 2015). Incorporation of blackcurrant significantly increased the diameter and moisture and decreased hardness of the wholemeal cookies. This could be due to the higher mineral content of blackcurrant powder that reacted with the flour protein and starch-protein interaction to improve the cookie textural properties (Brennan & Samyue 2004; Mais &

Brennan 2008). The original pigment of blackcurrant powder significantly increased the redness of the cookie colour (chapter 4). Incorporation of astaxanthin-rich microalgae *H. pluvialis* into cookies decreased weight and decreased diameter, which could be due to the acid-base and hydrophilic nature of the components (Okpala et al. 2013). The physical state of starch, fibre and protein of different flours and microalgae contributed to the textural properties of cookie (Yamsaengsung et al. 2012; Inglett et al. 2015). The increased colour to redness of the cookie was possibly due to the pigment of microalgae powder. The sugars and amino acids reaction (dextrinization and caramelization) enhances the darkness of the cookie colour during baking (Chevallier et al. 2000) (chapter 4-5).

Wholemeal cereal cookies exerted the highest glucose release, whereas greater control of glucose release as shown in blackcurrant incorporated (combination of dietary fruit and fibre) cookies (Chapter 4) as well as the astaxanthin-rich microalgae incorporated cookies even though they had less fibre content than cookie control (Chapter 5). The results suggested that functional molecules other than fibre, particularly polyphenols may have had an impact in attenuating the enzymatic digestion, and/or polyphenols and polysaccharides may act in a synergistic fashion. This observation have been recorded by previous research (Jakobek 2015). Anti-diabetic effects of wholegrain cereals consumption has been reported in observational studies in the past decades, which is regulated by the dietary fibre content of cereals and/or cereal products (McKeown et al. 2002; Liese et al. 2003). Incorporation of blackcurrant and astaxanthin-rich microalgae in wholemeal cookie significantly increased the fibre content that might build a non-starchy network of fibre and protein that impair the enzyme accessibility (Wolter et al. 2014). Moreover, interaction between phenolic compounds and digestive enzymes decreased starch degradation during *in-vitro* digestion (Bordenave et al. 2014). However, this potential health benefit is influenced by multiple gastrointestinal factors, food matrix and biofunctional components available in foods that influence the postprandial glycaemia, metabolic and endocrine responses (Nielsen et al. 2014; Marcadenti 2016). Simulated (multi-phase models with sequential addition of gastrointestinal enzymes under physiological conditions) digestion is a physiologically relevant, ethical, time and cost effective method in order to reveal

metabolic effects on the human body. High-glycaemic load (rapidly digested starches, particularly cereal products) has provided a means of causing the extent to raise blood glucose level, which may determine the risk of diabetes (Edwards et al. 2015). Although, the literature suggested that high fibre is positively associated with insulin sensitivity is somewhat mixed, the specific combination of dietary fruit and fibre enhances glycaemic control in our study.

An imbalance between pro-oxidants and antioxidants is one of the primary pathogenic mechanisms to the progression of obesity, CVD and cancer (Bar-Or et al. 2015). Existing research supports the beneficial effects of dietary fruits and fibre in antioxidant activity and inflammatory process (Anhê et al. 2015; Alissa & Ferns 2017). Bioactive phenolics can contribute to physiological functions, act as antioxidants, to protect the human body. The significantly higher TPC values observed in blackcurrant/microalgae incorporated cookie methanolic extracts (chapter 4-5) and digesta (chapter 8-9), antioxidant dietary fibre (chapter 10-11) and fermented metabolites (chapter 7) when compared with cookie control. The higher TPC content of the cookie is likely due to the higher phenolics of blackcurrant (14-18 mg/g) and microalgae (10-40 mg/g) (Kähkönen et al. 2001; Spiller & Dewell 2003). Ferulic acid, *p*-hydroxybenzoic, *p*-coumaric acid, kaemferol-3-glucoside and quercetin-3-rutinoside were the most abundant phenolic acids in all blackcurrant incorporated cookies (chapter 8 and 10), which are the identified potent antioxidant compounds. Cinnamic and salicylic acids were identified in astaxanthin incorporated cookie, indicating that *H. pluvialis* contributed significantly to the total phenolic acids of cookies (chapter 9 and 11), this observation is in agreement with previous research (Safafar et al. 2015). Quercetin and *p*-coumaric acid have been shown to be highly effective on the OH group reducing properties compared with cinnamic acid (Rice-Evans et al. 1996; Carrasco-Pozo et al. 2016). Although free phenolics are widely distributed in a plant food, a significant amount of fruit and cereals phenolics are composed of densely packed complex formation of polysaccharides and polyphenols (bound phenolics, chapter 10 and 11), which are inaccessible to gastrointestinal enzymes, but released in the lower digestive tract and may enhance gut health and prevent colon cancer.

In the cell-free systems (reported in chapter 8-11) radical scavenging assays were used to study antioxidant capacity of the components, before further study were undertaken in cell lines. Chemical antioxidant capacity assays particularly ORAC and DPPH are simple, sensitive, rapid and generally accepted methods to evaluate the components radical scavenging capacity, which reflect the relevant biofunctional attributes of foods. Total antioxidant capacity was higher in blackcurrant and microalgae incorporated cookies digesta, antioxidant-DF extracts, fermented metabolites and phenolic extracts compared to the control. Blackcurrant and microalgae provided additive interactions in the cookies and significantly contributed to enhance the peroxy radical scavenging capacity (chapter 4-5 and 7-11), and these observations were positively correlated to the higher phenolic content. However, not all dietary phenolics have high antioxidant properties. The reverse phase-HPLC identified the major phenolic acids in the blackcurrant/microalgae incorporated cookies, particularly ferulic acid, quercetin, kaempferol-3-glucoside and carotenoids might be responsible for the potent antioxidant capacity (chapter 8-11). This may lead to the significant differences observed in blackcurrant/microalgae incorporated cookie when compared with the cookie control.

Chemical antioxidant capacity assays do not adequately reflect the real biological value of the compounds, as several endogenous factors and inherent mechanisms may interfere with their bioavailability at cellular level. CAA assay are more relevant to complex biological systems and anti-oxidation mechanisms. The cellular antioxidant capacity of cookie digesta, antioxidant-DF extracts of cookie, fermented metabolites and cookie phenolic extracts, expressed by quercetin equivalents, were significantly higher in HepG2 cell model. The higher CAA values of blackcurrant/microalgae incorporated cookies (chapter 8-11), suggests that the bioactive compounds available in the cookies, are highly bioavailable and physiologically stronger to inhibit oxidation within the cells. The same samples also exerted high antioxidant capacity at the same ratio in PBS wash protocol, indicating the cellular uptake and bioactivity of phenolic compounds. Moreover, the antioxidant capacity of the blackcurrant and astaxanthin cookies might be a complex mixture of other nutrients, which independently or synergistically attenuates free radical scavenging capacity.

Food intake regulation is one of the most important factors involved in increasing obesity. Appetite modulation through alterations in the physiology of regulating gut hormones has been well characterised and investigated as anti-obesity therapy. A suite of anorexigenic hormones, including glucagon-like peptide-1 (GLP-1), cholecystinin (CCK), gastrin-releasing peptide (GRP), peroxisome proliferator-activated receptor γ (PPAR γ) and nucleobindin-2 (NUCB2)/nesfatin-1 have been identified as regulatory peptide hormones to suppress appetite in humans. They are synthesized and released in response to nutrient (Perry & Wang 2012). Administration of cookie digested metabolites and/or extracts in cell model lead to a significant up-regulation of GLP-1, CCK, GRP, PPAR γ and NUCB2 mRNA expression, indicating anti-obesity utility. These anorexigenic effects were shown to be mediated in response to the cookie metabolites, which were able to triggering the signals. Importantly, circulating levels of CCK and GLP-1 are influenced by diet composition and respond within 1 h post-feeding (Steinert et al. 2016). Appetite suppression in response to administration of cookie extracts or metabolites have provided an indication for this result in previous studies. Circulating CCK, GLP-1 and PPAR γ levels are lower in obese subjects suggesting a causative role in the development of obesity (Chambers et al. 2015; Steinert et al. 2016). It is noteworthy that anorexigenic effects of these regulatory hormones have been shown in normal weight as well as obese individuals (Perry & Wang 2012).

Alzheimer's disease is the most common neurodegenerative disease which is characterized by the aggregation of A β . While genetic and environmental interactions have been identified to account for most AD cases (Mattson 2004; Van Cauwenberghe et al. 2016), this study was designed to investigate the hypothesis that the bacterial metabolites from the gut might play an anti-inflammatory role in a manner that could attenuate A β -aggregates in a cell model of AD. SCFAs can cross the BBR, impact the cellular biological mechanism in the CNS and alter neural function (Mitchell et al. 2011). The cells treated with higher concentration of bacterial produced SCFAs accelerated A β -aggregation compared to control medium, suggesting that overproduction of SCFAs influence amyloidgenic disorder in the cell model (chapter 12). Consistent with this result, recent study have demonstrated gut metabolites promote A β pathology in murine model of AD (Minter et al. 2016). Dietary habit, composition of gut

bacteria and the SCFAs ratios including elevated level of particular SCFAs (acetate, propionate and butyrate) might play a role in neurodegeneration (Hooper et al. 2012; Unger et al. 2016). The tantalizing link of SCFAs to neurodegeneration may extend the hypothesis that gut-brain interactions represent unrevealed aetiology for AD. Identification of gut bacteria or their metabolites that are altered in AD may serve as markers and lead to interventions which may halt the progression of AD. Taken together, this study increases our understanding of the triangle connection between bioactive food components, metabolic and homeostatic control. Thus, biofunctional molecules could be a new direction in multifactorial approach to obesity therapy and homeostatic control. The intensive investigation of this study including glycaemic control, antioxidation, and gut and inflammatory markers beneficially affected by the bioactive compound in cookie certainly holds great promise to anti-obesity, anti-diabetic and anti-cancer therapy. *In-vitro* bacterial fermentation and their metabolites reveals the possible links between food intake and modulation of brain and gut health. Exerted synergistic effects of bioactive fibres and polyphenols may be a new and effective option to prevent and control chronic diseases in human. Indeed, microbial metabolite (to cell model of AD promotes A β -aggregation) represents a risk factor for amyloidogenic neurodegeneration. However, the exact molecular mechanisms linking nutrition remain elusive.

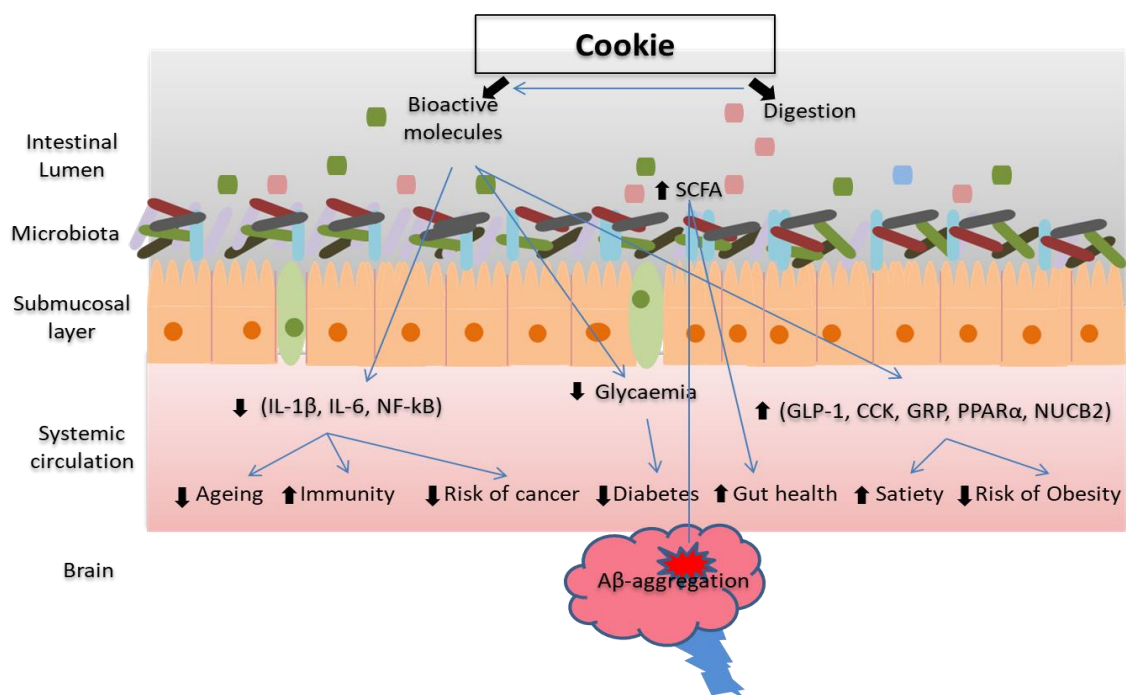


Figure 13.1. Predicted health benefits of cookie metabolites to the host

Future perspective

Food is a major contributor to health and diseases. In addition to extensive research on food intakes for health promotion and prevention of nutrition-related diseases, it is not well documented and characterised for the differences in particular dietary compounds which are causative or promote or are unrelated to the pathophysiology. Importantly, gut microbiota plays an important role in immune, metabolic, epigenetic regulation and neurodegenerative processes. While research over the past few years demonstrated that differences in microbial community are linked to certain clinical conditions, the physiological role of individual bacteria to the host is not characterised. It is obvious to address Question such as: are the effects of diet on the changes in the microbial community? Are changes in the microbial community underlying the pathological condition or a result thereof? Are there microbial taxa and/or microbially produced metabolites that are important for protective neural health need to be addressed. Understanding the interaction between dietary compounds, gut modulation and host pathophysiology should be the future research challenge. The use of animal models could be a great tool for understanding the basic processes in health and diseases.

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