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Cytochemical assessment of brewers' spent grain (BSG) extract bioactivities.

Thesis presented by

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For the degree of

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents.

Signed: _____

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Publications

Research papers

1. Crowley D, O'Callaghan Y, McCarthy A, Connolly A, Piggott CO, FitzGerald RJ, O'Brien, NM. Immunomodulatory potential of a brewers' spent grain protein hydrolysate incorporated into low-fat milk following *in vitro* gastrointestinal digestion. *Int J Food Sci Nutr* (2015) **66**, 672–676.

2. Crowley D, O'Callaghan Y, McCarthy AL, Connolly A, FitzGerald RJ, O'Brien NM. Aqueous and enzyme-extracted phenolic compounds from brewers' spent grain (BSG): Assessment of their antioxidant potential. *J Food Biochem* (2017) **41**, e12370.

Review paper

1. Crowley D, O'Callaghan Y, O'Brien NM. The Immunomodulatory Potential of Cereal Grains. (Review) *Curr Nutr Food Sci* (2017) **13**.

Abstracts

1. Crowley DJ, O'Callaghan YC, McCarthy AL, Piggott CO, FitzGerald RJ, O'Brien NM. The immunomodulatory potential of an *in vitro* digested low-fat milk supplemented with brewers' spent grain protein hydrolysate; selection of a non-toxic level of digestate. *Proc Nutr Soc* (2015) **74**, OCE1.

2. Crowley DJ, O'Callaghan YC, McCarthy AL, Connolly A, FitzGerald RJ, O'Brien, NM. Bioactivity of phenolic extracts of Brewers' Spent Grain (BSG) - assessment of their DNA protective effect against oxidant-induced DNA single strand breaks in U937 cells. *Proc Nutr Soc* (2015) **74**, OCE4.

3. Crowley DJ, O'Callaghan YC, McCarthy AL, Connolly A, FitzGerald RJ, O'Brien, NM. Cellular antioxidant and apoptotic potential of brewers' spent grain (BSG) phenolic extracts. *44th Annual Food Research Conference* (2015) P42.

4. Crowley DJ, O'Callaghan YC, Connolly A, FitzGerald RJ, O'Brien, NM. Effect of enzyme-extracted brewers' spent grain protein hydrolysates on inflammatory response in cells associated with atherosclerosis. *Proc Nutr Soc* (2016) **75**, OCE3.

Abstract

Brewers' spent grain (BSG) is a useful source of protein and phenolic compounds which can be obtained via different extraction methods. The aim of this thesis was to assess protein hydrolysates and phenolic extracts generated from BSG using various extraction procedures, for their potential as functional food ingredients with anti-inflammatory, antioxidant, anti-cancer and neuroprotective properties.

Firstly, the anti-inflammatory effects of an alkaline-extracted BSG protein rich fraction and three ultrafiltration-generated fractions added to milk and subjected to simulated gastrointestinal digestion (SGID) was shown in Jurkat T cells.

Enzyme extraction methods were then used to produce phenolic fractions with strong bioactivities. The cellular antioxidant potential of both black and pale BSG phenolic extracts isolated using carbohydrases was demonstrated in U937 cells and HepG2 cells. Following this, the ability of the most active phenolic extracts to enhance the antioxidant potential of flavoured water drinks before and after SGID was investigated. However, none of the phenolic extracts added to water drinks significantly increased antioxidant activity. In other studies the anti-cancer potential of a pale BSG phenolic extract was demonstrated, through the increase of apoptosis in U937 cells. Additionally, the neuroprotective potential of both black and pale BSG phenolic extracts was shown in SK-N-BE(2) neuronal cells, where extracts protected against hydrogen peroxide (H_2O_2)-induced cytotoxicity, H_2O_2-induced apoptosis and H_2O_2-induced lipid peroxidation.

Finally, the cytotoxicity, anti-inflammatory activity and antioxidant potential of BSG protein hydrolysates generated using different extraction procedures was assessed and compared. Direct enzymatic hydrolysates demonstrated anti-inflammatory activity in Jurkat T cells and RAW 264.7 cells. Alkaline-extracted BSG protein hydrolysates, as well as a 10kDa permeate showed anti-inflammatory effects in RAW 264.7 cells. As well as this, unhydrolysed fractions, a direct enzymatic hydrolysate, alkaline-extracted BSG protein hydrolysates, as well as a 10kDa permeate also displayed cellular antioxidant effects in U937 cells and HepG2 cells.

Abbreviations

5-LOX	5-Lipoxygenase
γ-ORZ	Gamma oryzanol
AACC	American Association of Cereal Chemists
AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride
ABTS+	2,20-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
ACE	Angiotensin-converting enzyme
AD	Alzheimer's disease
Alc	Alcalase
ALS	Amyotrophic lateral sclerosis
ANOVA	One-way analysis of variance
AP-1	Activator protein 1
AX	Arabinoxylans
AXOS	Arabinoxylan oligosaccharide
BCA	Bicinchoninic acid
BGEE	Beta-glucan-enriched extract
BMDCs	Bone Marrow-Derived Dendritic Cells
BSG	Brewers' spent grain
BSY	Brewers' spent yeast
CAF	Cycloartenyl *trans*-ferulate
CAT	Catalase
CCl_4	Carbon tetrachloride
CIS	Cerebral ischemic stroke

ConA	Concanavalin A
CorPP	Corolase PP
COX-2	Cyclooxygenase-2
CRBC	Chicken red blood cells
CRP	C-reactive protein
DH	Degree of hydrolysis
DHAvD	Dihydroavenanthramide D
DMEM	Dulbecco's Modified Eagle Medium
DPP4	Dipeptidyl peptidase-4
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DSM	Dutch State Mines
DSS	Dextran sulphate sodium
DW	Dry weight
ECACC	European Collection of Authenticated Cell Cultures
EECB	Ethanolic extract of corn bran
FBRA	Fermentation of brown rice and rice bran
FBS	Fetal bovine serum
FCR	Folin–Ciocalteu reagent
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
$FeSO_4$	Ferrous sulphate
Flav	Flavourzyme
FO	Feruloylated oligosaccharide

FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalents
GSH	Glutathione
GI	Gastrointestinal
H_2O_2	Hydrogen peroxide
HAD	Hydroxycinnamic acid derivative
HAEC	Human aortic endothelial cells
HBSS	Hank's Balanced Salt Solution
HCA	Hydroxycinnamic acid
HD	Huntington’s disease
HMR	Hydroxymatairesinol
HMR2	7-Hydroxymatairesinol 2
HNE	4-Hydroxynonenal
HT-29	Human intestinal epithelial
IC_{50}	Inhibitory concentration-50
ICAM-1	Intercellular adhesion molecule 1
IFN-γ	Interferon gamma
IκB-α	Inhibitor of NFκB-α
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
LDL	Low density lipoprotein
LOX-1	Lectin-type oxidized LDL receptor 1
LPS	Lipopolysaccharide

MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein 1
MDA	Malondialdehyde
MetOH	Methanol
MMP	Mitochondrial membrane potential
MMP-9	Matrix metallopeptidase 9
MRP	Maillard browning reaction products
MS	Multiple sclerosis
MTT	(3 -(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NaOH	Sodium hydroxide
NBb	Njavara ‘black glumed’ type medicinal rice
NBr	Njavara rice
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NRU	Neutral red uptake
ORAC	Oxygen radical absorbance capacity
OVA	Ovalbumin
PAI-1	Plasminogen activator inhibitor-1
PD	Parkinson’s disease
PGE_2	Prostaglandin E2
PMBC	Peripheral blood mononuclear cells
Ppar-γ	Peroxisome proliferator-activated receptor gamma
Pro	Prolyve 1000

Pro	Protease P
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acids
RAGE	Receptor for advanced glycation end-products
RBL	Rat basophilic leukemia
RMR	Red mold rice
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SE	Standard error
SGID	Simulated gastrointestinal digestion
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TE	Trolox equivalents
TGF-β	Transforming growth factor beta
Th2	Type 2 helper T cells
TLR	Toll-like receptor
TNBS	Trinitrobenzenosulfonic
TNF-α	Tumor necrosis factor alpha
TPA	12-O-Tetradecanolylphorbol-13-acetate
TPC	Total phenolic content
TTGE	4’-O-(erythro-β-guaiacylglyceryl) ether
VCAM-1	Vascular cell adhesion molecule 1
VEGF-α	Vascular endothelial growth factor alpha

VEWs	Vitamin enhanced waters
v/v	Volume/volume
WPCH	Whey protein concentrate hydrolysates
w/v	Weight/volume

Chapter 1

Literature review

The Immunomodulatory Potential of Cereal Grains

Published in: *Current Nutrition and Food Science* (2017) **13**.

Abstract

Inflammation is a normal response to tissue injury or infection and many chronic diseases are characterised to some extent by the presence of low-grade inflammation. Considerable interest exists in reducing chronic disease risk or managing illnesses through health-promoting dietary ingredients. Cereals are a source of macronutrients, minerals, vitamins and other micronutrients and are also known to contain a range of biologically active substances such as arabinoxylans, β-glucans, cellulose, lignans, lignin, sterols, tocopherols, tocotrienols, alkylresorcinols, phenolic acids and microelements. Cereal grains displaying anti-inflammatory and immunomodulatory activity may have an important role to play in alleviating some of the primary symptoms of inflammation. This paper reviews some of the recent research conducted on cereal grains in relation to their anti-inflammatory and immunomodulatory activity.

Introduction

Cereal grains and cereal grain products are staple constituents of the diet in many parts the world (Gani *et al*., 2012). Cereals are a source of macronutrients, minerals and vitamins (Carnevali *et al*., 2014). A range of biologically active substances such as arabinoxylans (AX), β-glucans, cellulose, lignans, lignin, sterols, tocopherols, tocotrienols, alkylresorcinols, phenolic acids and microelements are also found in cereal grains (Bartlomiej *et al*., 2012). It is recognised that, as well as their nutrient profile, cereals have the potential to enhance health and that cereal grain consumption can considerably lower diet-related disease risk (Topping, 2007).

The most common cereals are wheat, corn (maize), rice, barley, oats, sorghum, millet and rye. They are grown on almost 60% of cultivated land globally. Botanically, cereals are classed as grasses and belong to the monocot family *Poaceae*. Wheat, rye and barley are closely related, being part of the subfamily

Pooideae and the tribus *Triticeae*. Oats are categorized as a distant relative of the *Triticeae*, within the subfamily *Pooideae*, whereas rice, corn, sorghum and millet exhibit separate evolutionary lines. Buckwheat, wild rice, quinoa and amaranth are not classed as cereal grains, but often called pseudo-cereals owing to their similar composition and usage (Slavin, 2004; Frolich and Aman, 2010). Within each cereal species numerous varieties exist produced by breeding in order to optimize agronomical, technological and nutritional properties.

Compositionally, cereals comprise of 12-14 percent water, 65-75 percent carbohydrates, 2-6 percent lipids and 7-12 percent protein. Cereals show similarities regarding gross composition, being low in protein and high in carbohydrates (Table 1). Oats and maize however contain substantial amounts of lipids. Various cultivars of a given type of cereal show compositional variability.

The anatomy of cereal grains is quite uniform. Grains have a protective component known as the hull or husk containing the endosperm, bran and germ. Bran (fruit and seed coats) surrounds the germ and the endosperm and consists of the starchy endosperm and the aleurone layer. In oats, barley and rice the husk is attached together with the fruit coat and cannot be directly removed by threshing as seen with common wheat and rye. The chemical components of cereals are not evenly distributed in the grain (Table 2). The husk and bran are high in cellulose, pentosans and ash. The aleurone layer of wheat contains more minerals than the endosperm; whereas the lipids are generally found in the aleurone and germ. The endosperm, which mainly consists of starch, has lower protein content than the germ and the bran, and is low in fat and ash.

The European Union HEALTHGRAIN consortium defines whole grains as ‘Whole grains shall consist of the intact, ground, cracked or flaked kernel after the removal of inedible parts such as the hull and husk. The principal anatomical components – the starchy endosperm, germ and bran – are present in the same relative proportions as they exist in the intact kernel’ (Van der Kamp *et al*., 2014). In refined grains, the milling process removes the bran and germ fractions (Gani *et al*., 2012).

Table 1: Proximate composition of cereal grains[1]

Cereal	Crude protein	Crude fat	Ash	Crude fibre	Available carbohydrate
Wheat	10.6	1.9	1.4	1.0	69.7
Brown Rice	7.3	2.2	1.4	0.8	64.3
Maize	9.8	4.9	1.4	2.0	63.6
Barley	11.0	3.4	1.9	3.7	55.8
Oats	9.3	5.9	2.3	2.3	62.9
Sorghum	8.3	3.9	2.6	4.1	62.9
Rye	8.7	1.5	1.8	2.2	71.8
Pearl Millet	11.5	4.7	1.5	1.5	63.4

[1] *percent dry weight;* (Alais and Linden, 1991)

Table 2: Distribution of major components of wheat grain

Fraction	Proportion	Protein	Lipid	Minerals
Whole grain	100	12	2.0	2.0
Endosperm	80	10	1.2	0.6
Aleurone	8	18	8.5	15.0
Bran	8.5	6	1.0	3.5

[1] *percent dry weight;* (Alais and Linden, 1991)

Major Cereal Grains - Wheat, Rice, Maize and Barley

Wheat, rice, maize and barley, are among the principal cereal grains produced worldwide (Gani *et al*., 2012). Rice is an important staple food, particularly in Asia (Choi *et al*., 2010). There are a number of different rice types such as long-grain white, long-grain brown, glutinous brown, wild, basmati, brown basmati, jasmine and risotto. According to Chen *et al.* (2010), a number of components from rice have different pharmacological and biological activities. Maize is one of the most important crops in the world and is consumed in a number of forms such as sweet corn, cooked kernels, tortillas, chips and polenta (Fardet *et al*., 2008). As discussed by Choi *et al.* (2013), barley is an important cereal grain and is grown as a human food source, however 80-90% of barley produced is consumed by animals (Fardet *et al*., 2008). Barley is more popular as a human food in Eastern compared to Western societies (Slavin *et al*., 2000).

Lesser Cereal Grains - Oats, Rye, Millet, Sorghum, Buckwheat, Wild Rice and Amaranth

Oats are usually consumed as a wholegrain cereal (Fardet *et al*., 2008) and are also extensively studied as a dietary source of the soluble fibre β-glucan, which has been shown to reduce elevated blood glucose, triglyceride and cholesterol (Ahmad *et al*., 2014). Rye is mostly consumed in Scandinavian countries, particularly in the form of whole-grain rye bread (Fardet *et al*., 2008). Sorghum is a cereal mostly consumed in semi-arid regions of the world, although human consumption is limited (Fardet *et al*., 2008). Sorghum is a source of phenolic compounds, including phenolic acids, flavonoids and proanthocyanidins (Dykes and Rooney, 2006).

Diet and Inflammation

Inflammation is a normal response to tissue injury or infection (Bullo *et al*., 2007). Many chronic diseases are characterised to some extent by inflammation. The inflammatory process is initiated by the production and secretion of pro-inflammatory cytokines which may be triggered by an inflammatory insult (e.g. a poor, unbalanced diet) (Bullo *et al*., 2007; Lee *et al*., 2013a). The increased

cytokine synthesis and the subsequent increase in reactive oxygen and nitrogen species are key elements of the inflammatory process. This process is controlled by a negative feedback mechanism and is in turn followed by anti-inflammatory cytokine secretion to counter the increase of reactive species. Likewise, the cellular anti-oxidant defence system is also initiated to curb the progression of chronic inflammation where the risk of metabolic syndrome and subsequently atherosclerosis is greatly increased (Bullo *et al*., 2007).

Dietary habits are likely to be one of the principal determinants of the balance that influences inflammation in chronic conditions. Genetic polymorphisms at numerous sites may influence the ability of bioactive food components to affect inflammation by modulating pro- and/or anti-inflammatory mediators (Bullo *et al*., 2007). There is a lot of interest in reducing chronic disease risk or managing illnesses through health-promoting dietary ingredients. The health-promoting properties of cereal grain consumption in lowering the risk of chronic diseases is the focus of many research laboratories (Masisi *et al*., 2016). Cereal grains displaying anti-inflammatory and immunomodulatory activity may have an important role to play in alleviating some of the primary symptoms of inflammation.

This review attempted to summarise recent findings on the anti-inflammatory and immunomodulatory components of different cereal grains. The primary objective of the review was to report on the latest evidence supporting the bioactivity of various cereal grain extracts, bran, aleurone, phenolic compounds, β-glucan, AX and peptides.

Anti-inflammatory effects of cereal grain extracts

Considerable evidence on the anti-inflammatory potential of cereal grains has been garnered from studies assessing various grain extracts. Different extraction methods have been applied such as ethanol, methanol and aqueous extraction. In many published studies however, the bioactive components have not been definitely identified.

Several studies have investigated the anti-inflammatory potential of extracts prepared from rice using various extraction procedures. An ethanolic extract of

whole grain, black rice inhibited lipopolysaccharide (LPS)-induced expression of pro-inflammatory mediators, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) in RAW 264.7 mouse macrophage cells through inhibition of the nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) signalling pathway (Limtrakul *et al*., 2015). Further analysis showed that the extract demonstrating anti-inflammatory activity was rich in the flavonoid, anthocyanin, and also hydroxybenzoic acid. An ethanolic extract of black rice was also found to reduce eosinophil infiltration and Type 2 helper T cell (Th2) cytokines and decrease airway inflammation, as evidenced by lung histological examination, in an ovalbumin (OVA)-induced mouse model of chronic asthma (Lee *et al*., 2006). The black rice extract also decreased Th2 cytokines in cultured splenocytes and bronchoalveolar lavage fluid in a concentration-dependent fashion. The dried hull of rice is a by-product of the rice milling process. Kim *et al.* (2011) generated a liquid smoke extract of dried rice hulls and found that the extract reduced nitric oxide (NO) production in LPS-induced RAW 264.7 mouse macrophage leukemia cells and decreased TNF-α, IL-1β and IL-6 in 12-O-tetradecanolylphorbol-13-acetate (TPA)-induced edema in CD-1 mice. The bioactive compounds in the extract were not identified. Both a methanolic and an aqueous extract of red rice demonstrated a concentration-dependent inhibition of IL-1β, IL-6 and COX-2 mRNA expression in LPS-stimulated RAW 264.7 macrophages (Niu *et al*., 2013). The red rice extracts were shown to have a high phenolic content. Overall, these data suggest that rice is a good source of anti-inflammatory compounds and given that it is one of the most consumed grains worldwide, the impact on health benefits are likely to be significant.

Delipidated wheat, rye, buckwheat, oat and corn aqueous extracts, were shown to increase levels of the anti-inflammatory cytokine IL-10 in peripheral blood mononuclear cells (PBMCs) obtained from healthy individuals; the IL-10 secretion by PBMCs led to the inhibition of interferon gamma (IFN-γ), IL-5 and IL-13 production in T-cells (Yamazaki *et al*., 2008). An extract prepared from proso millet L. (*Panicum miliaceum*), using ultra-sonication, was fed to ob/ob (obese) mice at a concentration of 1% diet and was found to decrease serum levels of the cytokines, monocyte chemotactic protein 1 (MCP-1), IL-6 and TNF-α in

the mice (Park *et al*., 2011). An aqueous extract prepared from the thread-like strands on the ears of corn (corn silk) demonstrated anti-inflammatory activity during acute inflammation in a carrageenan-induced pleurisy rat model. Pre-treatment with 2g/kg and 4g/kg body weight of corn silk extract significantly attenuated TNF-α, IL-1β, vascular endothelial growth factor alpha (VEGF-α), IL-17, NF-κB activation and iNOS protein expression in a dose-dependent manner (Wang *et al*., 2012). Ethanolic extracts from buckwheat decreased inflammation in ethanol- and carbon tetrachloride (CCl_4)-treated C57BL mice and Sprague-Dawley rats. The major active components, identified as quercetin and rutin, were also administered to the test animals. The results indicate that the buckwheat extract and the individual flavonoids, quercetin and rutin countered the effects of ethanol- and CCL_4-induced liver damage by a number of mechanisms including increasing antioxidant enzyme activity and inhibiting inflammatory cytokines (Lee *et al*., 2013b). In a similar study, male Sprague-Dawley rats fed with an ethanolic extract of buckwheat (*Fagopyrum cymosum*) had less severe clinical arthritis compared with the control group, as shown by reduced paw swelling and lower polyarthritis index. Serum levels of IL-1 and TNF-α were also reduced in the extract-treated group (Shen *et al*., 2013). The ability to inhibit enzymes involved in arachidonic acid metabolism has been identified as a potential mechanism of action of a number of plant extracts traditionally associated with the treatment of inflammatory and cardiovascular diseases. A butanolic extract prepared from oats was found to inhibit arachidonic acid metabolism in human blood platelets through an inhibition of the cyclooxygenase and lipoxygenase pathways (Ahmed *et al*., 2013). This effect was not observed in oat fractions prepared using water and hexane. In summary, these studies demonstrate positive effects of extracts prepared from a variety of grains including wheat, rye, buckwheat, oat and corn in cell, animal and human studies.

Recent years have seen an increase in the consumer demand for cereal sprout products as the germination of grains may increase the nutritional and functional properties of the food (Cornejo *et al*., 2015). Manukumar *et al.* (2014) studied the impact of germination time on the antioxidant and anti-inflammatory activity of sorghum grains and found that a methanolic extract of 48h-germinated sorghum grain demonstrated better hemolysis protection compared to the raw sorghum

extract in human erythrocytes. The authors associated the bioactivity of these extracts with their polyphenol content. An ethanolic extract of buckwheat sprouts demonstrated anti-inflammatory activity in a human intestinal epithelial cell line, CoLoTC cells, as determined by a reduction in LPS-stimulated IL-8 release (Ishii *et al*., 2008). The authors also reported an anti-inflammatory effect in mice using both oral and intraperitoneal LPS-treatment. The oral administration of buckwheat sprouts decreased IL-6 and TNF-α in the spleen and liver of LPS-administered mice. The mechanism involved was unclear and the authors could not identify a relationship between anti-inflammatory activity and the six flavonoids which were detected in the buckwheat sprouts. The anti-inflammatory effect of ethanolic extracts prepared from buckwheat sprouts were also assessed in LPS-stimulated RAW 264.7 macrophages and compared with the flavonoid, rutin (Karki *et al*., 2013). The authors reported a significant inhibition of both COX-2 expression and the translocation of NF-κB p65 subunit in the nuclear fraction by buckwheat sprouts. They suggest that the buckwheat sprouts may interfere with the interaction of LPS with toll-like receptor (TLR)-4 in the cell culture model utilised**.** From the limited studies in this area, it appears that sprouting may enhance the amounts of anti-inflammatory compounds in certain grains. It is an interesting area and certainly warrants further investigation.

Cereals and cereal components may be used in functional food formulations as fermentable substrates to promote the growth of probiotic microorganisms or as sources of dietary fibre providing a number of physiological benefits (Das *et al*., 2012). Fermentation of cereal grains may therefore be a useful technique for the generation of bioactive compounds. The fermentation of brown rice and rice bran (FBRA) with *Aspergillus oryzae* prevented large bowel shortening in a mouse model for human familial adenomatous polyposis (Phutthaphadoong *et al*., 2010). FBRA administration significantly decreased iNOS and COX-2 mRNA expression in colonic tissue of the treated mice. FBRA also decreased cell proliferation in colonic crypt cells. Red mold rice (*Monascus*-fermented rice) generates many functional secondary metabolites. Cheng and Pan, (2011) demonstrated the hepatoprotective effects of *Monascus*-fermented red mold rice (RMR) using a chronic alcohol-induced C57BL/6J mouse model. RMR supplementation attenuated the production of hepatic IL-6, IL-1β and TNF-α. In

addition, it was noted that RMR decreased hepatic transforming growth factor beta (TGF-β) production. Lin *et al.* (2011) showed that TNF-α-treated human aortic endothelial cells (HAEC) supplemented with different *Monascus*-fermented rice metabolites, exhibited lower adhesion with human monocytic cells U937; the effect of the metabolites was dose-dependent. *Monascus*-fermented rice metabolites also ameliorated TNF-α-induced vascular cell adhesion molecule 1 (VCAM-1) and E-selectin protein expression. The proposed mechanism involved a suppression of NF-κB activation, preventing TNF-α-induced VCAM-1 and E-selectin expression in HAECs, leading to a reduction in the monocyte adhesiveness to endothelial cells. In summary, research suggests that fermentation enhances the amount of anti-inflammatory compounds in cereal grains. Additional research is necessary to fully assess the potential value of this approach from a human health perspective.

A number of different extraction methods have been used to obtain cereal fractions with anti-inflammatory activity. Phenolic compounds have been identified as active compounds in a number of these studies. Future research should focus on optimising these extraction methods and identification of other compounds within these grains that offer anti-inflammatory activity. More information on the mechanisms involved in the anti-inflammatory activity is also necessary. In addition, few clinical trials on the anti-inflammatory potential of cereal grains have been carried out. Clinical trials would be beneficial to further substantiate and confirm the anti-inflammatory potential of cereal grains.

Anti-inflammatory effects of various cereal fractions

Extracts prepared from whole grains demonstrate anti-inflammatory activity in various model systems as reviewed above. Distribution of bioactive compounds in the grain fractions is therefore of interest. Wheat aleurone is an example of novel wheat grain fraction with high levels of potentially health-promoting compounds. This sections reviews studies on anti-inflammatory activity of bran, aleurone and phenolic fractions from various grains and on the anti-inflammatory potential of grain derived β-glucans and AX.

Bran Fraction

The bran is the hard outer layers of the grain surrounding the germ and endosperm which protects the grain from the external environment (Slavin, 2004). Most bioactive compounds of whole-grain cereal grains are present in the bran/germ fraction (Gani *et al*., 2012). A number of studies have been published on the anti-inflammatory potential of cereal bran fractions.

Rye bran is an excellent source of dietary fibres and phytochemicals and is a potential prebiotic. Håkansson *et al.* (2009) demonstrated that rye bran, both alone and in combination with blueberry husks, altered the dextran sulphate sodium (DSS)-inflammatory response in Sprague-Dawley rats by reducing the oxidative damage caused by reactive oxygen species (ROS) involved in the induction of colitis. The polyphenolic components of rye bran and blueberry husks may have been responsible for reducing oxidative damage. Oat bran consumption reduced soluble VCAM-1 in low density lipoprotein (LDL)-receptor-deficient mice (Andersson *et al*., 2010). Additionally, mice fed a typical Western-type diet (Research Diets Inc., NJ, USA) with the addition of oat bran (40% w/w) demonstrated reduced levels of fibrinogen. However, inclusion of oat bran in the diet of these animals had no impact on plasma concentrations of IL-6. The authors speculated that the effect on inflammatory markers may be more significant postprandially, rather than in the fasted state. They also suggested that oat components such as avenanthramides, vitamin E and other phenolic compounds may have been responsible for the anti-inflammatory effects. Wheat bran is also a good source of fatty acids, tocopherols, polysaccharides and phenolic compounds (Akhtar *et al*., 2012). Belobrajdic *et al.* (2011) assessed the effects of wheat bran on inflammation and oxidative stress in lean and obese Zucker rats. They found that in obese rats, the wheat bran diet decreased plasminogen activator inhibitor-1 (PAI-1) levels in plasma but had no effect on leptin, IL-1β or IL-6. Hosoda *et al.* (2012) assessed the effects of methanolic extracts from the bran of foxtail millet, barnyard millet and proso millet on NO and inflammatory cytokine production in LPS-stimulated RAW 264.7 macrophages. Proso millet bran extract demonstrated the strongest inhibitory effect against LPS-induced NO production and LPS-induced IL-6 secretion at 0.25-0.5% extract. Foxtail and proso millet bran extracts showed stronger inhibitory effects on LPS-stimulated TNF-α secretion compared

to barnyard millet bran extract. Again, the anti-inflammatory effects of the bran extracts were associated with the phenolic content of the brans.

Rice bran contains ferulic acid and is also high in dietary fibres. It is currently used to treat hyperlipidaemia, hypertension and obesity (Mizushina *et al*., 2013; Park *et al*., 2013). Many studies have investigated the anti-inflammatory effects of rice bran. The anti-inflammatory activity of methanolic extracts of bran from Njavara 'black glumed' type medicinal rice (NBb) and its corresponding rice (NBr) in a carrageenan-induced paw edema in rats was assessed by Mohanlal *et al.* (2013). At a dose of 5mg/kg body weight, NBb inhibited edema after 3 and 5 h to a greater extent compared to NBr and other staple rice varieties. An enzymatic extract of rice bran reduced vascular inflammation by decreasing aortic iNOS and TNF-α expression when consumed by obese Zucker rats (Justo *et al*., 2013). Similarly, Candiracci *et al.* (2014) investigated the impact of dietary supplementation of an enzymatic rice bran extract on adipose tissue inflammation markers in obese Zucker rats. The extract decreased the expression of TNF-α, IL-6, IL-1β, and iNOS in the adipose tissue of these obese rats. Choi *et al.* (2010) assessed the anti-inflammatory effects of black rice bran (cv. LK1-3-6-12-1-1) against TPA-induced skin edema and 2, 4-dinitrofluorobenzene-induced allergic contact dermatitis in inflammatory mouse models. Intraperitoneal treatment with pigmented black rice bran ethanol extract, prior to TPA application in CD-1 mice, decreased neutrophil levels and the production of TNF-α, IL-1β and IL-6 in ear tissue. Black rice bran extract also decreased arachidonate 5-lipoxygenase (5-LOX) mRNA and protein expression in ear tissue. Suppression of 5-LOX expression by black rice bran extract was thought to be linked to the down-regulation of intercellular adhesion molecule-1 (ICAM-1) expression which contributes to a blockade of inflammatory neutrophil migration into the tissue and to the subsequent aggravation of inflammation induced by the infiltrated cells. A recently-developed enzyme-treated rice bran prebiotic, composed of dietary fibre (>70%) and a fat-soluble fraction decreased proinflammatory cytokines (TNF-α, IFN-γ, IL-1β, IL-6, IL-12p70) and anti-inflammatory cytokines (IL-4) in DSS-treated female BALB/c mice (Komiyama *et al*., 2011). Additionally, the hydrophilic fraction (ethanol-methanol soluble fraction) of the prebiotic demonstrated the ability to down-regulate dendritic cell proliferation. The authors

speculated that the anti-inflammatory effects of the prebiotic were partly as a result of microbiota modulation and increasing short chain fatty acid production.

Mizushina *et al.* (2013) found that a component of the by-product of sake-brewing, cycloartenyl *trans*-ferulate (CAF), demonstrated anti-inflammatory activity in a CAF suppressed TPA-induced inflammation in the mouse ear but ferulic acid did not. There was a larger quantity of CAF in the outer pericarp of sake-brewing rice compared to ordinary rice. Glycoproteins play a significant role as structural constituents of the cell wall in many plants. The glycoprotein fraction from rice bran (GFRB) was assessed in LPS-stimulated RAW 264.7 cells by Park *et al.* (2013) and was found to increase IL-6, IL-10, IL-1β and TNF-α in RAW 264.7 cells. The authors speculated that glycans are likely to be the active component in GFRB, as treatment of hydrolysed GFRB with a combination of amylase and pectinase reduced the anti-inflammatory effect.

The bran fraction of cereal grains is a valuable source of compounds with anti-inflammatory activity. Considering that bran, along with the germ fraction, is usually removed during the milling process, retaining this fraction for use, for example, in functional food formulation, would be worthwhile given the potential it has in treating inflammatory symptoms.

Aleurone Fraction

According to the American Association of Cereal Chemists (AACC), the aleurone is defined as "the outermost layer(s) of the endosperm in cereal grains, and surrounds the starchy endosperm and part of the embryo. Cereal aleurone tissue is separated from the germ and starchy endosperm by standard milling practices, starting with the grain kernel or starting with the bran, followed by further extraction processes'' (AACC, 2010). Aleurone-rich fractions lowered pro-inflammatory mediators to a greater extent than crude fractions of wheat bran in a mouse model of obesity (Neyrinck *et al*., 2008). A prebiotic made from the aleurone layer and scutellum fractions of barley malt, and containing glutamine-rich and hemicellulose-rich fibre, reduced colitis in immune-deficient female mice (Kanauchi *et al*., 2008).

Mateo Anson *et al.* (2010) digested wheat fractions (aleurone, bran and flour) using an *in vitro* model, simulating the upper gastrointestinal (GI) tract. Dialysates were retrieved from jejunal and ileal sections at 1 hour intervals and represented the bioaccessible components from digested wheat fractions. The aleurone fraction was the only fraction to demonstrate an anti-inflammatory effect following 3-4 hr digestion as determined by a suppressed LPS-stimulated TNF-α production in U937 macrophages. This would indicate that the aleurone may be the most suitable wheat fraction to provide a sustained release of anti-inflammatory compounds during gastrointestinal digestion. The inclusion of wheat aleurone in the diet of apparently healthy, older, overweight men and women produced a small but significant decrease in the fasting blood concentration of the inflammatory marker, C-reactive protein (CRP) (Price *et al*., 2012). The authors suggested that the observed CRP-lowering activity may be due to a number of potentially bioactive components present in aleurone, which may function individually or synergistically.

Similar to bran, the aleurone component of cereal grains holds potential as a source of bioactive compounds that can be used to target inflammation. Further research is necessary to fully clarify and identify the anti-inflammatory compounds in this portion of the grain.

Phenolic Fraction

Phenolic compounds are secondary metabolites which play a role in the growth and reproduction of plants, as well as in plant defence mechanisms and pigmentation (Gani *et al*., 2012). Phenolics are concentrated in the bran fraction of the cereal grain where they are present in either the free, soluble conjugated or insoluble forms bound to arabinosyl chains of the cell wall (De la Fuente *et al*., 2011). Phenolic acids are one type of phenolic compound found in cereals and consist of two main classes: hydroxybenzoic acids and hydroxycinnamic acids. Flavonoids are another class of phenolic compounds and are found in the pericarp (Dykes and Rooney, 2007). Phenolic acids and flavonoids are among the most ubiquitous phenolic compounds in whole-grain cereals. They are thought to act as stabilisers within the cell wall structure, as well as playing a role in the defence

against microorganisms. The amount of phenolic acids present in cereals is dependent upon the variety and type of cereal as well as the growing conditions and the time of harvest (Andersson *et al*., 2014). Among cereal grains, sorghum and millet have the greatest variety of phenolic acids (Dykes and Rooney, 2007). Phenolic compounds may be unique to particular cereal grains, for example tricin in rice and avenanthramides in oats, whereas certain phenolic acids, such as ferulic and cinnamic acid are found in a diverse range of cereals and other plants. Table 3 summarises the various methods reported for measuring and identifying the anti-inflammatory phenolics of cereal grains.

Whent *et al.* (2012) demonstrated that whole wheat flours contained phenolics, as well as lutein and α-tocopherol, and reported that ethanol extracts from these wheat flours had both anti-inflammatory and anti-proliferative activity in cell culture models. In particular, an extract from a WestBred 936 wheat cultivar significantly inhibited IL-1β mRNA expression in mouse J774A.1 macrophages. Methanol extracts from the aerial parts of barley showed anti-inflammatory effects in LPS-stimulated RAW 264.7 macrophages and in a septic mouse model (Choi *et al*., 2013). Gas chromatography-mass spectrometry analysis demonstrated the presence of derivatives of benzoic and cinnamic acids and fatty acids in the extracts. Brewers' spent grain (BSG), a by-product of the brewing industry has been highlighted as a useful source of phenolic compounds. McCarthy *et al.* (2014) demonstrated that two phenolic extracts from BSG generated via sodium hydroxide (NaOH) extraction, caused significant alterations in inflammatory cytokine production in concanavalin A (ConA)-stimulated Jurkat T cells. The extracts contained high levels of hydroxycinnamic acids. In a follow-up study, food digestates containing the phenolic extracts from BSG, altered cytokine production in ConA-stimulated Jurkat T cells (McCarthy *et al*., 2015).

A number of phenolic compounds with anti-inflammatory potential have also been identified in the bran fraction of various cereals. An aqueous maize husk extract demonstrated an anti-inflammatory effect in rats which was associated with the content of tannins and polyphenols in the extracts (Owoyele *et al*., 2010). Hole *et al.* (2012) identified the main free and bound phenolic acids in methanol and hydrolysed extracts of wheat, barley and oat flour as ferulic, caffeic,

Table 3: Anti-inflammatory activity of phenolic compounds from cereal grains

Cereal grain	Phenolic compound	Model	Anti-inflammatory effect	Reference
Wheat (flour)	Ferulic acid	LPS-stimulated J774A.1 macrophages	Inhibited IL-1β mRNA expression	Whent *et al*., 2012
Barley	Derivatives of benzoic acid and cinnamic acid	LPS-stimulated RAW 264.7 macrophages and LPS-injected septic mouse model	Suppression of NO, iNOS, TNF-α, IL-6, IL-1β and DNA-binding activity of NF-κB. Improved survival rate of LPS-injected mice.	(Choi *et al*., 2013)
Brewers' spent grain	Ferulic acid, *p*-coumaric acid and caffeic acid	ConA-stimulated Jurkat T cells	Reduced IL-2, IL-4, IL-10 and IFN-γ production	McCarthy *et al*., 2014
Brewers' spent grain (food digestates)	Ferulic acid, *p*-coumaric acid and caffeic acid	ConA-stimulated Jurkat T cells	Increased IL-4 production. Decreased IL-10 and IFN-γ production	McCarthy *et al*., 2015
Maize (husk extract)	Tannins and polyphenols	Cotton pellet granuloma and carrageenan-induced paw edema in rats	Inhibition of paw edema and granuloma formation	Owoyele *et al*., 2010

Oat, barley and wheat (flour)	Ferulic acid, caffeic acid, *p*-coumaric acid and sinapic acid	LPS-stimulated U937-3κB-LUC cells	Inhibition of NF-κB activity	Hole *et al*., 2012
Pearl millet	Polyphenols	Splenic T-cells from Wistar rats	Decreased PMA- and Ionomycin-induced MAPK phosphorylation and IL-2 mRNA expression. Increased intracellular free calcium	Nani *et al*., 2015
Adlay (bran)	Syringaldehyde, vanillic acid, ferulic acid, protocatechuic acid, caffeic acid, *p*-coumaric acid and luteolin	A23187-stimulated rat basophilic leukemia (RBL)-2H3 cells	Suppressed mast cell degranulation. Decreased IL-4, IL-6 and TNF-α secretion. Reduced ROS production, phosphorylation of Akt and expression of protein kinase C	Chen *et al*., 2012
Maize (bran)	*p*-Dicoumaroyl putrescine, diferuloylputrescine, *p*-coumaric acid and ferulic acid	LPS-stimulated RAW 264.7 macrophages	Decreased NO production and iNOS expression	Kim *et al*., 2012
Sorghum	Benzoic acid and cinnamic acid derivatives	LPS-stimulated RAW 264.7 macrophages	Inhibited NO production. Decreased iNOS and COX-2 expression	Nguyen *et al*., 2015
N/a	Morin	MSU crystal-induced acute gouty arthritis	Decreased secretion of TNF-α, IL-1β, IL-6, MCP-1, VEGF and PGE_2. Lowered NF-κB p65 and p-NF-κB p65 expression	Dhanasekar *et al*., 2016

		in Wistar albino rats		
N/a	Lignan (hydroxymatairesinol and 7-hydroxymatairesinol 2)	TNF-α-stimulated HAECs	Inhibited TNF-α-stimulated ICAM-1 and VCAM-1 protein expression in HAEC. Reduced U937 cells adhesion to HAECs	Spilioti *et al*., 2014
Rye	Alkylresorcinols	Human lymphocytes	Increased lymphocyte subpopulation numbers	Gasiorowski *et al*., 2000
Rice (Koshihikari)	γ-Oryzanol, cycloartenyl ferulate and ferulic acid	DSS-induced colitis in C57BL/6J mice	Suppressed a number of inflammatory parameters including TNF-α, IL-1β and IL-6 transcription and COX-2 expression	Islam *et al*., 2008
Rice (Njavara)	Tricin	LPS-stimulated human PMBCs	Inhibited COX-2 and iNOS expression. Down-regulated TNF-α, IL-6, PGE_2 and NO production. Inhibited MMP-2 and MMP-9 activation and NF-κB signalling	Shalini *et al*., 2012
Rice (Njavara)	Tricin	LPS-stimulated RAW 264.7 macrophages and TPA-induced ear edema in ICR mice	Decreased ROS, NO and PGE_2. Inhibited iNOS and COX-2 expression and NF-κB DNA-binding activity. Reduced TPA-induced ear reddening in mice	Jung *et al*., 2014
Rice (bran)	Feruloylated oligosaccharides	Murine BMDCs	Induced IL-12 production in BMDCs. Promoted dendritic cell maturation. Induced NF-κB signalling pathways	Lin *et al*., 2014

Oats	Avenanthramides	IL-1β-stimulated HAECs	Decreased expression of adhesion molecules, chemokine and pro-inflammatory cytokine production and HAEC adherence to U937 cells	Liu *et al*., 2004
Oats	Avenanthramides	TNF-α-induced keratinocytes and resiniferatoxin-induced ear edema in ICR mice	Inhibited NF-κB, p65 activation and NF-κB luciferase activity. Inhibited neurogenic inflammation in mice	Sur *et al*., 2008
N/a	Avenanthramides (dihydroavenanthramide D)	Cytokine-induced RINm5F insulinoma cells, isolated Sprague–Dawley rat islets and STZ-treated ICR mice	Prevented NF-κB activation	Lv *et al*., 2009
Oats	None*	TNF-α-induced human 293T cells	Inhibited NF-κB activation	Chu *et al*., 2013
Oats	Avenanthramides	TNF-α-stimulated mouse myoblast	Inhibited NF-κB activation	Yang *et al*., 2014

		C2C12 cells		
Oats	Avenanthramides	Postmenopausal women undertaking downhill walking	Decreased exercise-induced neutrophil respiratory burst activity, plasma CRP concentration, IL-1β levels and mononuclear cell NF-κB activation	Koenig *et al*., 2014

N/a, not assessed. * no correlation between phenolic content and anti-inflammatory activity.

p-coumaric and sinapic acids. The authors then showed that a combination of these phenolic acids modulated LPS-induced NF-κB activity in the U937-3κB-LUC cell line. Millet polyphenols demonstrated immunosuppressive effects in splenic T-cells from Wistar rats via the inhibition of IL-2 mRNA expression and mitogen-activated protein kinase (MAPK) phosphorylation (Nani *et al*., 2015).

Ethyl acetate-soluble fractions of ethanolic extracts of adlay bran decreased IL-4, IL-6 and TNF-α secretion in rat basophilic leukemia (RBL)-2H3 cells (Chen *et al*., 2012). Using 1-D nuclear magnetic resonance and mass spectroscopy spectral analysis, the authors identified a number of phenolic acids (syringaldehyde, vanillic acid, ferulic acid, protocatechuic acid, caffeic acid, and *p*-coumaric acid) and one flavone (luteolin) present in the adlay bran. With the exception of ferulic acid and caffeic acid, all of these compounds demonstrated an ability to suppress mast cell degranulation activity. Kim *et al.* (2012) found that an 80% ethanolic extract of corn bran (EECB) significantly repressed NO production and iNOS expression in a dose-dependent fashion in LPS-stimulated RAW 264.7 macrophages. All four hydroxycinnamic acid derivative (HAD) components found in EECB (*p*-dicoumaroyl putrescine, diferuloylputrescine, *p*-coumaric acid and ferulic acid) attenuated NO production and iNOS expression in a dose-dependent fashion.

There are a number of studies in which individual phenolic acids were isolated from cereals and their anti-inflammatory activity was assessed. Benzoic acid and cinnamic acid derivatives isolated from methanolic extracts of sorghum displayed potential inhibitory effects against NO production in LPS-induced RAW 264.7 cells (Nguyen *et al*., 2015). Morin (2′,3′,4′,5,7-pentahydroxyflavone), a bioflavonoid found in cereals, showed anti-inflammatory effects in a Wistar albino rat model of acute gouty arthritis (Dhanasekar *et al*., 2016) as determined by decreased levels of TNF-α, IL-1β, IL-6, MCP-1, VEGF and prostaglandin E_2 (PGE_2) in serum.

Lignans are plant polyphenols which have demonstrated positive health benefits. Spilioti *et al.* (2014) found that hydroxymatairesinol (HMR) – a lignan, and its major isomer 7-hydroxymatairesinol 2 (HMR2) were effective at blocking TNF-α-induced ICAM-1 and VCAM-1 expression in HAECs. HMR is found in wheat, triticale, oats, barley, millet, corn bran and amaranth whole grain.

Alkylresorcinols are also a class of phenolic compounds found in whole grain cereals (Gani *et al*., 2012). Gasiorowski *et al.* (2000) isolated and purified the alkylresorcinols (5-n-alk (en) ylresorcinols) from a milled rye bran fraction and demonstrated the immunomodulatory action of alkylresorcinols in human lymphocyte cultures.

γ-Oryzanol (γ-ORZ) is comprised of phytosteryl ferulates, made up of 24-methylenecycloartanyl ferulate, CAF, β-sitosteryl and campestanyl ferulate. Oral supplementation with γ-ORZ, purified from the Japanese rice, Koshihikari, decreased TNF-α, IL-1β and IL-6 transcription and also decreased COX-2 expression in DSS-induced colitis in mice (Islam *et al*., 2008).

The flavonoid, tricin, was isolated from Njavara rice bran, and was found to attenuate LPS-stimulated PGE_2 and NO production in human PMBCs through the inhibition of COX-2 and iNOS expression (Shalini *et al*., 2012). Subsequently, Jung *et al.* (2014) examined the anti-inflammatory effects of a methanol-extracted tricin, 4'-O-(erythro-β-guaiacylglyceryl) ether (TTGE), from Njavara rice bran in LPS-treated RAW 264.7 macrophages and in an experimental edema model in mice. TTGE dose-dependently decreased ROS, NO and PGE_2 in LPS-stimulated RAW 264.7 macrophages. Additionally, TTGE decreased the ear reddening induced by TPA in mice. Lin *et al.* (2014) demonstrated the stimulating effect of feruloylated oligosaccharides (FOs) isolated from rice bran on dendritic cells and also found that FOs induced IL-12 production in bone marrow-derived dendritic cells (BMDCs).

Avenanthramides are the principal phenolic antioxidants found in oats (Liu *et al*., 2004). The main role of avenanthramides is thought to relate to host protection as they exhibit anti-microbial activity (Jung *et al*., 2014). Avenanthramides range from 40-132 μg/g in cereal grains (Gani *et al*., 2012). Liu *et al.* (2004) reported that pre-treatment of HAECs with an ethanol-extracted avenanthramide-enriched mixture decreased IL-1β-stimulated HAEC expression of adhesion molecules, chemokine and pro-inflammatory cytokine production and HAEC adherence to U937 cells in a dose-dependent manner. In another study, the pre-treatment of keratinocytes with avenanthramides prevented degradation of inhibitors of NFκB-α (I*κ*B-α) (Sur *et al*., 2008). It was also shown that topical application of avenanthramides inhibited 0.05% resiniferatoxin-induced neurogenic

inflammation in ICR mice (Sur *et al*., 2008). A study assessing dihydroavenanthramide D (DHAvD), the synthetic form of avenanthramide, demonstrated that DHAvD protected pancreatic β cells (RINm5F insulinoma cells and isolated Sprague–Dawley rat islets) from the toxic effects of cytokines (Lv *et al*., 2009). However, there was no correlation between avenanthramide levels in whole oat groats and NF-κB inhibitory activity in human 293T cells, suggesting that other components are involved in the anti-inflammatory activity (Chu *et al*., 2013). Yang *et al.* (2014) demonstrated the anti-inflammatory activity of three oat avenanthramides in TNF-α-stimulated NF-κB activation in mouse myoblast C2C12 cells and noted that variation in the NF-κB-suppressive effects of the three avenanthramides were related to their structural differences. Avenanthramide supplementation decreased exercise-induced neutrophil respiratory burst activity, plasma CRP concentration, IL-1β levels and mononuclear cell NF-κB activation in in postmenopausal women (Koenig *et al*., 2014).

It is clear that phenolic compounds present in cereal grains may play an important role in the attenuation of inflammation. Considering also, that the amount of phenolic compounds present in cereals depends on the variety and type of cereal grain, for example, tricin in rice and avenanthramides in oats, there may be scope to focus on certain cereal types and manipulate growing conditions in order to maximise the phenolic compound yield.

β-Glucans

β-Glucans are large, viscous, water-soluble cell wall constituents (soluble fibre) present in certain cereal grains, including oats (Rieder *et al*., 2011; Fardet *et al*., 2008). β-Glucan possesses a number of functionalities and roles that make it unique as a plant cell wall component. There are a limited number of studies which indicate that β-glucan has immunomodulatory properties.

CD3+ T cells co-cultured with dendritic cells which had been exposed to the barley polysaccharide, MSK-BG (a pure, linear (1→3), (1→4)-β-glucan) demonstrated increased IFN-γ and IL-4 secretion (Chan *et al*., 2007). The authors proposed that MSK-BG may be used to compliment antibody-based

targeted therapy in cancer treatments. Barley β-glucans from whole barley flour had a more pronounced inhibitory effect on TNF-α-stimulated VCAM-1 expression in HAECs, compared to oat β-glucans (Lazaridou *et al*., 2011). This was associated with structural differences - barley β-glucans chains have greater levels of 3-O-β-cellobiosyl-D-glucose portions (β-(1 → 3) glycosyl linkages compared to oats. Rieder *et al.* (2011) investigated the effects of commercial, cereal-derived β-glucans with different average molecular weights on cytokine secretion in IL-1α-stimulated Caco-2 cells and TNF-α-stimulated human intestinal epithelial cells (HT-29 cells). In HT29 cells, IL-8 secretion increased in response to 40kDa and 359kDa β-glucans, whereas Caco-2 cells were unresponsive. The authors speculated that slight differences in structure and composition, outside of size, may account for differences in immuno-stimulatory activity observed for the different samples. In a more recent study, treatment with a commercially available β-glucan-enriched extract (BGEE) in streptozotocin-induced diabetic rats was investigated (Uskokovic *et al*., 2013). BGEE treatment led to the normalization of the serum concentrations of the "positive" and "negative" acute-phase proteins, α 2 -macroglobulin and albumin, respectively. In addition, the expression of anti-inflammatory cytokines IL-10 and IL-4 was also upregulated. The researchers demonstrated that BGEE elicited its effects by impeding the receptor for advanced glycation end-products (RAGE)/NF-κB signalling pathway.

β-Glucan is known for its ability to attenuate elevated blood glucose, triglyceride and cholesterol (Ahmad *et al*., 2014). However, there is a limited amount of evidence suggesting that it plays an important role in reducing inflammation and further investigation is warranted.

Arabinoxylans

AX are non-starch polysaccharides, mainly consisting of xylose and arabinose (Bartlomiej *et al*., 2012; Mendis and Simsek, 2014). Cereal processing practices such as milling, gluten-starch separation and brewing result in AX-rich by-products. AX and its hydrolysis products arabinoxylan oligosaccharides (AXOS) have demonstrated different health benefits, including immunomodulatory activity

in the limited number of published studies (Mendis and Simsek, 2014). Thus, utilization of these industrial by-products for the extraction of health-promoting dietary fibers holds much promise for these industries (Mendis and Simsek, 2014). The full impact of AX with respect to human health has yet to be explored.

Two water-soluble AX, which were sequentially extracted from wheat bran using enzymatic or NaOH extraction were investigated for their complement-fixing activity using sensitized red blood cells from sheep (Hromadkova *et al*., 2013). The complement-fixing activity of the enzyme-extracted fraction was lower than that of the NaOH-extracted fraction. Furthermore, the wheat bran fraction extracted using NaOH performed better than the positive control, the immunogenic polysaccharide PM II. Stimulation with a purified polysaccharide subfraction, BLE-P-I, which was isolated from barley leaf using pectinase, led to a significant increase in the macrophage-stimulating cytokines TNF-α, IL-6, and IL-12 in RAW 264.7 cells (Kim *et al*., 2016). Purification by column chromatography established that glucuronoarabinoxylan (40–45%) and rhamnogalacturonan-I mainly branched with a type II arabinogalactan side chain (30–35%) were the predominant polysaccharides contained in the extract.

The anti-inflammatory effect of water-soluble rice bran polysaccharides and sulfated rice bran polysaccharides from defatted rice bran was assessed in mouse melanoma cell line B16 and RAW 264.7 macrophages (Wang *et al*., 2016). It was found that the fraction derived from the polysaccharide, RBP2 - an arabingalactan consisting mainly of galactose and arabinose, and the sulfated polysaccharide, SRBP2 - derived from RBP2, showed immune-enhancing activity in RAW 264.7 cells by increasing NO and TNF-α secretion. AX also increased the macrophage phagocytic activity of chicken red blood cells (CRBC) in S-180 tumor-bearing ICR mice and also promoted ConA and LPS-stimulated splenocyte proliferation in tumor-bearing mice (Cao *et al*., 2011). In addition, AX increased the effectiveness of natural killer cells from splenocytes in these mice, indicating the potential to increase the cytotoxic activity against tumor cells (Cao *et al*., 2011). Similarly, AX from wheat bran, extracted using NaOH, stimulated antibody responses in Hubbard chickens (Akhtar *et al*., 2012). Enzyme-extracted AX and alkaline-extracted AX, from de-starched wheat bran, were orally administered to

mice and were shown to increase ConA- and LPS-stimulated splenocyte proliferation (Zhou *et al*., 2010).

The ability of a granular foodstuff containing hydrolysed rice bran to prevent the common cold syndrome in elderly people was investigated by Maeda *et al.* (2004). The hydrolysed rice bran was a water-soluble dietary fibre fraction extracted from rice bran and was found to contain AX as the main constituent. The rice bran extract reduced the duration of common cold symptoms and decreased the severity of symptoms and the requirement for symptomatic therapy, showing its value in decreasing physical stress at the acute phase of respiratory infection. AX have a complex structure compared to other commonly studied dietary fibres and understanding the structure-function relationship between AX and immunomodulatory potential needs further research.

AX has considerable potential as an anti-inflammatory compound. From a functional standpoint, AX is an easily extractable grain component produced during cereal processing. It may therefore provide a useful immunomodulatory fraction that can be added to functional foods for human consumption.

Anti-inflammatory effects of peptides from cereal grains

The protein content of cereals ranges from approximately 7% to 11% of dry weight and cereals are the primary source of protein for a large part of the world's population. Cereal protein is a good source of essential amino acids, with the exception of the limiting amino acids lysine, threonine and methionine, and is also a source of bioactive peptides which may be released from the parent protein during food processing or during gastro-intestinal digestion by the action of either digestive enzymes or colonic bacteria (Korhonen and Pihlanto, 2006). Cereal peptides have documented health benefits including angiotensin-converting enzyme (ACE) inhibition (Cian *et al*., 2015), facilitating a reduced glycaemic response to carbohydrate intake (Ishikawa *et al*., 2015) and antioxidant effects (Zhu *et al*., 2006; Chanput *et al*., 2009; Yin *et al*., 2015). Peptides derived from many foods such as milk, meat, eggs and plant sources, including cereals, have also shown anti-inflammatory effects (Chakrabarti *et al*., 2015). The mechanisms underlying the anti-inflammatory effects of food-derived peptides have not been

fully elucidated and can vary depending of the peptide structure or sequence (Majumder *et al*., 2013).

Glutein and prolamin are the major protein classes in cereals. Prolamins have a high proline and glutamine content and, in particular, wheat prolamins (gluten) have a unique amino acid profile which consists of greater than 40% glutamyl residues (Shewry *et al*., 1986). The prolamin fraction contains the peptide sequences responsible for the allergenicity of cereals however, a number of anti-inflammatory peptides have also been derived from the prolamin fraction (Silano *et al*., 2008; De Vita *et al*., 2012). The prolamin fraction of rice was identified as the fraction with the highest cytokine-mediated anti-leukemic response, in an *in vitro* study, in comparison with the albumin, globulin, and glutelin fractions (Chen *et al*., 2010).

Several investigators have demonstrated that peptides derived from cereals including wheat and maize can protect against inflammatory bowel disorders such as ulcerative colitis and celiac disease (Giordani *et al*., 2014; Mochizuki *et al*., 2010). Celiac disease is characterised by intestinal inflammation resulting from exposure to prolamins, particularly the gliadin fraction of wheat. Despite its inflammatory effects in celiac disease, wheat gluten has also been shown to contain peptide sequences capable of moderating the inflammatory effects of gliadin. A decapeptide (QQPQDAVQPF), which had previously been identified in durum wheat (De Vincenzi *et al*., 1997), was synthesised by Silano *et al.* (2008) and was found to ameliorate the wheat gliadin-induced inflammatory response in T-cells obtained from celiac patients. The proposed mechanism was a binding of the decapeptide to the antigen presenting cells thereby inhibiting presentation of the antigen to T-cells and the subsequent inflammatory response. A substitution of glycine for aspartic acid at position 5 or alanine at position 6 diminished the anti-inflammatory activity of the decapeptide indicating the significance of the peptide sequence and structure in this anti-inflammatory effect (Silano *et al*., 2008). An analogous peptide sequence (QQPQRPQQPF), also identified in cereals, similarly demonstrated protection against inflammation associated with celiac disease (De Vita *et al*., 2012). A more recent study found that both these decapeptides hindered the up-regulation of pro-inflammatory cytokines by human dendritic cells, which are potent antigen presenting cells, as well as their ability to induce T

cell proliferation when dendritic cells were pre-incubated with the peptides prior to exposure to gliadin (Giordani *et al*., 2014). The oral administration of an enzymatic hydrolysate of maize gluten (zein) reduced the mucosal levels of inflammatory markers, histamine and myeloperoxidase activity, in a rat model of inflammatory bowel disease (Mochizuki *et al*., 2010) and may therefore be beneficial in the treatment or prevention of Crohn's disease and ulcerative colitis.

Pryoglutamyl peptides, a product of the heat treatment of glutamine, have been identified in wheat gluten and appear to be resistant to gastrointestinal digestion (Higaki-Sato *et al*., 2003). Both free and peptide forms of pyroglutamic acid have been detected in the plasma of rats following the intragastric administration of wheat gluten hydrolysate indicating that they are bioavailable (Higaki-Sato *et al*., 2003). Hirai *et al.* (2014) investigated the anti-inflammatory effects of pyroglutamyl-leucine in an LPS-stimulated mouse macrophage model and compared it with the dipeptides pyroglutamyl-leucine, pyroglutamyl-valine, pyroglutamyl-methionine and pyroglutamyl-phenylalanine. Each of the dipeptides was found to reduce the secretion of pro-inflammatory cytokines in LPS-stimulated mouse macrophages in a dose-dependent manner. The most marked effect was observed for pyroglutamyl-leucine and the authors investigated the mechanism involved in the anti-inflammatory effect of this dipeptide. They found that the dipeptide did not inhibit binding of LPS to the cell surface of macrophages but exerted its anti-inflammatory effect through a suppression of IκBκ degradation and MAPK phosphorylation and inhibition of NF-κB activation. The authors proposed that the ability of pyroglutamyl-leucine to moderate inflammation downstream of LPS-TLR-4 binding would indicate that the dipeptide is transported into cells where it directly interacts with inflammatory pathways.

One of the processes contributing to the progression of multiple sclerosis (MS) is the inflammatory demyelination of cells of the nervous system (Lisak and Hohlfeld, 2007). A 16 amino acid peptide (AEMIDALAAKMLSEGRG) termed RA1, identified as a fragment of cooked Japanese rice, was found to reduce inflammation in a mouse model of MS (Shapira *et al*., 2010). In addition, when these animals were orally administered an extract of Japanese cooked rice they also demonstrated a significant reduction in neurological symptoms. The authors

speculated that the presence of this peptide in rice may contribute to the lower incidence of MS in Japan and China. This peptide was also found to be synthesised by T regulatory cells in response to incubation with a novel anti-inflammatory peptide IIIM1, a nonapeptide derived from histone H2A. RA1 also reduced the oxidative burst of activated macrophages (Schussheim *et al*., 2011) which is a fundamental activity of many known anti-inflammatory agents. As stated by the authors, the fact that RA1 was active following oral administration indicates that the peptide is bioavailable and may also be effective against other chronic inflammatory disorders (Shapira *et al*., 2010).

Protein hydrolysates derived from BSG have demonstrated anti-inflammatory potential by their ability to reduce ConA-stimulated IFN-γ production in Jurkat T cells (McCarthy *et al*., 2013). BSG incorporated into a variety of foodstuffs and subjected to an *in vitro* digestion procedure has also demonstrated anti-inflammatory potential in cell culture models (Crowley *et al*., 2015; McCarthy *et al*., 2015). Montoya-Rodriguez *et al.* (2014a) showed that extruded amaranth hydrolysates significantly reduced TNF-α secretion in both LPS-induced THP-1 and RAW 264.7 cells. This was accompanied by a reduction in PGE_2 and COX-2, along with a decrease in the expression of p65 NF-κB subunits in the cell nuclei. A subsequent study assessed the ability of pepsin-pancreatin hydrolysates from unprocessed and extruded amaranth to reduce atherosclerosis markers in LPS-induced THP-1 human macrophage-like cells (Montoya-Rodriguez *et al*., 2014b). Extruded amaranth hydrolysate demonstrated anti-inflammatory activity by reducing the expression of a host of proteins associated with the lectin-type oxidized LDL receptor 1 (LOX-1) signalling pathway in LPS-induced THP-1 human macrophage-like cells. Synthetic, pure peptides derived from amaranth proteins also demonstrated anti-inflammatory potential by a reduction in the expression of LOX-1, ICAM-1 and matrix metallopeptidase 9 **(**MMP-9) in LPS-induced THP-1 human macrophage-like cells (Montoya-Rodriguez *et al*., 2015).

Rice bran protein hydrolysates, extracted by alkaline or enzymatic mechanisms, were fed to male Sprague-Dawley rats on a high carbohydrate-high fat diet and a decrease in mRNA expression of proinflammatory cytokines, IL-6, TNF-α, iNOS and MCP-1 in the intra-abdominal fat cells was observed (Boonloh *et al*., 2015). The authors speculated that the decrease in these cytokines may be

related to the upregulation of peroxisome proliferator-activated receptor γ (Ppar-γ) which regulates fatty acid uptake and glucose metabolism. Additionally, the possibility that components in the extract other than peptides may enhance the bioactivity of the hydrolysate fractions was proposed.

There is a significant amount of evidence supporting that peptides from cereal grains have immunomodulatory activity. Cereal grain peptides may have potential as functional food ingredients aimed at reducing inflammation. More research is required, particularly with regard to the bioaccessibility and bioavailability of cereal grain proteins and peptides. There is also a need for further evaluation of the anti-inflammatory effects of cereal grain peptides in human populations to validate health claims.

Conclusion

In cereal crops the edible seed, referred to as the grain, has excellent nutritional qualities. Most bioactive compounds in whole-grain cereals are located in the bran/germ component (Gani *et al*., 2012). A number of different cereal grain components have shown anti-inflammatory and immunomodulatory activity, as demonstrated by the plethora of *in vitro* and *in vivo* studies which have been performed over the last decade. Many bioactive components have already been identified, whereas in some other cases, specific bioactive constituents of cereal grains have not yet been isolated. Some bioactive compounds are associated with specific cereals, for example γ-ORZ in rice, avenanthramides in oats, alkylresorcinols in rye and β-glucans in both oats and barley (Gani *et al*., 2012). Such compounds have the potential to become useful ingredients in 'functional foods' or foods/dietary components that contribute health benefits beyond basic nutrition (Das *et al*., 2012). Further work is however required to identify fully the specific bioactive components of cereal grains that elicit anti-inflammatory and immunomodulatory effects. Furthermore, greater numbers of studies using human subjects are required to validate fully the bioactivity of such components.

Another future challenge in the area of cereal grain research will involve detailed investigation of the bioaccessibility and bioavailability of bioactive constituents. Bioaccessibility is the first obstacle to a compound's bioavailability.

It relates to the intestinal release of a compound from a food matrix, making it available for absorption (Bjorck *et al*., 2012). This is particularly relevant to compounds, such as phenolics which are bound to the grain matrix, therefore hindering extraction and bioaccessibility (Dykes and Rooney, 2007). Different processing technologies have been created to modulate and increase the bioaccessibility of bound compounds such as phenolics in cereal grains (Wang *et al*., 2014). These include reducing particle size and degradation of cereal matrices or breakdown of fibre polymers via methods such as mechanical treatment, thermal treatment, bioprocessing and extrusion cooking. Such technologies may prove useful in the further exploitation of bioactive constituents present in cereal grains.

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Research Objective

Brewers' spent grain (BSG) is the insoluble portion of the barley malt leftover at the end of the brewing process and is known as a rich source of protein and phenolic compounds, with potential functional food applications. Phenolic extracts and protein hydrolysates generated using alkaline hydrolysis have previously demonstrated bioactive effects in *in vitro* cell culture models. The objective of this thesis was to investigate the bioactivity of phenolic extracts and protein hydrolysates generated from BSG using an extraction procedure utilising food-grade carbohydrase and proteinase enzymes, in *in vitro* cell culture models and to assess their potential as functional food ingredients.

Firstly, the anti-inflammatory activity of an alkaline-extracted protein-enriched isolate and subsequent fractions produced using membrane fractionation, which were added to low-fat milk and subjected to simulated gastrointestinal digestion (SGID), was investigated. The anti-inflammatory activity of the resultant digestates was assessed using an *in vitro* cell culture model.

Next, the antioxidant potential of enzyme-extracted BSG phenolic extracts was assessed in *in vitro* cell culture models. Following on from this, the most active phenolic extracts were then added to flavoured water, where the antioxidant activity of the drinks was measured before and after SGID.

Additionally, the anti-inflammatory and antioxidant activity of BSG protein hydrolysates was examined. Hydrolysates produced using direct enzymatic hydrolysis were assessed for their bioactive effects. Similarly, the bioactivity of another set of hydrolysates generated using direct enzymatic hydrolysis procedure was investigated and compared with hydrolysates produced using an alkaline-extraction method, alkaline-extracted hydrolysates subjected to SGID and alkaline-extracted hydrolysates generated using ultrafiltration.

The neuroprotective potential of BSG phenolic extracts was also researched, where the impact of extracts on oxidant-induced apoptosis and lipid peroxidation was measured. Finally, the antiproliferative effects of BSG phenolic extracts and protein hydrolysates in cells of a cancerous origin, was examined, as well as the ability of phenolic extracts to induce apoptosis.

Chapter 2

Immunomodulatory potential of a brewers' spent grain protein hydrolysate incorporated into low-fat milk following *in vitro* gastrointestinal digestion.

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Abstract

A brewers' spent grain (BSG) protein rich fraction was previously hydrolysed using Alcalase (U) and 3 additional fractions were prepared from U by membrane fractionation; a 5 kDa retentate (U >5), a 5kDa permeate (U <5) and a 3 kDa permeate (U <3). In the present study, these fractions were added to milk and subjected to simulated gastrointestinal digestion (SGID) and their anti-inflammatory potential was investigated. The digestates caused a significant reduction (P<0.05) in interleukin-6 (IL-6) production in Concanavalin-A (ConA)-stimulated Jurkat T cells. The samples did not significantly alter the production of IL-6 in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. IL-2 and interferon-γ (IFN-γ) production in stimulated Jurkat T cells and IL-1β and tumor necrosis factor-α (TNF-α) production in stimulated RAW 264.7 cells were also not affected in the presence of the digestates. The results show that a SGID milk product supplemented with BSG hydrolysate and its associated ultrafiltered fractions can confer anti-inflammatory effects in Jurkat T cells.

Introduction

The demand for 'healthy foods' has increased significantly over the last number of years (Hasler, 2002). This has led to the rapid increase in the development of functional foods containing bioactive food-derived ingredients or physiologically active nutrients with validated health benefits (Crowe and Francis, 2013). Protein hydrolysates from agricultural crops have demonstrated bioactive effects which support their potential use as functional food ingredients (McCarthy *et al*., 2013a; Celus *et al*., 2007).

One of the challenges to the commercialisation of bioactive protein hydrolysates is the limited amount of information available regarding their bioavailability which is the amount of an ingested nutrient that is absorbed and metabolised (Li-Chan, 2015). It has been estimated, for example, that up to 97% of the cereal-derived, anti-inflammatory peptide lunasin is destroyed by gastrointestinal digestion (Dia *et al*., 2009a). In addition, peptides can be released from the parent protein during the digestive process. A number of studies have reported the release of bioactive peptides following the simulated gastrointestinal digestion (SGID) of plant proteins (Lo *et al*., 2006; Marambe *et al*., 2011; Vermeirssen *et al*., 2005). SGID is typically used to determine the bioaccessibility of a nutrient and may be coupled with the Caco-2 cell model to estimate nutrient bioavailability (Ting *et al*., 2015) however, the *in vitro* SGID method has also been used to assess the stability of protein hydrolysates to gastrointestinal enzymes (Raikos and Dassios, 2014).

Inflammation is an essential process in the body's reaction to nonlethal injury, however, excessive and uncontrolled inflammatory responses can lead to chronic diseases (Chakrabarti *et al*., 2014). Several cereal-derived peptides have demonstrated anti-inflammatory effects. Shapira *et al*. (2010) found that a peptide derived from Japanese rice reduced the secretion of the pro-inflammatory cytokines interferon gamma (IFN-γ) and interleukin-17 (IL-17) and increased production of the anti-inflammatory cytokines IL-4 and IL-10 in splenocytes isolated from a mouse model of multiple sclerosis. Hydrolysates prepared from the pseudo-cereal amaranth have demonstrated anti-inflammatory effects in both lipopolysaccharide (LPS)-induced human THP-1 macrophage cells and RAW 264.7 cells (Montoya-Rodríguez *et al*., 2014). The peptide lunasin demonstrated

anti-inflammatory activity in LPS-induced RAW 264.7 cells (Dia *et al*., 2009b). Brewers' spent grain (BSG) which is the insoluble portion of the barley malt which remains at the end of the brewing process is a rich source of protein (Mussatto *et al*., 2006). Research from our laboratory has previously demonstrated that protein hydrolysates derived from BSG possess anti-inflammatory activity in human Jurkat T cells (McCarthy *et al*., 2013b).

In the present study a BSG protein-enriched isolate hydrolysed using Alcalase (U) and 3 fractions prepared from U using membrane fractionation; a 5 kDa retentate (U >5), a 5kDa permeate (U <5) and a 3 kDa permeate (U <3), were added to low-fat milk. The anti-inflammatory potential of these hydrolysates was previously reported in McCarthy *et al*. (2013b). The supplemented milk samples were then subjected to SGID and the anti-inflammatory potential of the resultant digestates was investigated in Concanavalin-A (ConA)-stimulated Jurkat T cells and LPS-stimulated murine macrophage RAW 264.7 cells. Both T cells and macrophages have a critical role in the immune response. T cells develop in the thymus and possess a T cell receptor on their surface. Macrophage cells originate from monocytes and engulf pathogens in addition to stimulating lymphocytes and other immune cells (Hu and Pasare, 2013). Con-A is a mitogenic lectin which stimulates the secretion of inflammatory cytokines in Jurkat T cells (Dupis and Bastin, 1988). RAW 264.7 cells are known to produce inflammatory cytokines in response to LPS and are widely used for the investigating the activity of potential anti-inflammatory compounds (Rossol *et al*., 2011).

Materials and Methods

Materials

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich Ireland Limited, Co. Wicklow, Ireland. Jurkat T cells and RAW 264.7 cells were obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK).

Preparation of protein hydrolysate

The BSG protein rich isolate was extracted at semi-pilot scale using a method previously described (Connolly *et al*., 2013). The parent hydrolysate (U) was prepared using Alcalase 2.4L. During hydrolysis, the pH was maintained by addition of NaOH using a pH-Stat system (Metrohm 718 STAT Titrino, Herisau, Switzerland) and the temperature was kept constant using a thermostatically controlled water bath (IKA® Werke GmbH & Co. KG, Staufen, Germany). The resulting hydrolysate was adjusted to pH 7.0, heated at 95°C for 10 minutes to inactivate the enzyme and was then freeze-dried (U) or fractionated using 5 and 3 kDa molecular cut-off membranes (Minimate™ Tangental Flow Filtration Capsules, Pall Corporation, New York, USA) prior to freeze drying (U >5, U <5, U <3).

Preparation of fortified food

The low-fat (1.5%) milk (Dawn Dairies, Co. Cork) was purchased in a local supermarket. Before addition to foods, a 1% (w/v) solution of freeze dried hydrolysate (U) or ultrafiltration fraction (U >5, U <5, U <3) solution was prepared using distilled, deionised water. Each of the hydrolysate samples (1%, w/v) were added to low-fat milk aliquots (50 mL) at a concentration of 0.125 % (v/v) to yield a milk product with a final concentration of 12.5 ppm sample, similar to previously published studies (McCarthy *et al*., 2015; Nasri *et al*., 2013; Segura-Campos *et al*., 2013).

Simulated gastrointestinal digestion (SGID)

A SGID model, as previously described (McCarthy *et al*., 2015) was utilised to simulate human digestion. Briefly, for the gastric digestion 2 mL sample and 1 mL Pepsin (0.04 g/mL in 0.1M HCl) were added to 12 mL Hank's balanced salt solution (HBSS) and the pH was adjusted to 2 using 0.1M HCl, to simulate the physiological conditions of the stomach. Samples were incubated for 1 h in a shaking waterbath (Grant OLS 200, Grant Instruments, Cambridge, UK) at 95 rpm and 37^{o}C. To simulate duodenal digestion, 100 µL Pancreatin (0.08 g/mL in HBSS) and 200 µL bile salts (0.2 g glycodeoxycholate, 0.125 g taurodeoxycholate

and 0.2 g taurocholate in 5 mL HBSS) were added, the pH was adjusted to 7.4 using 1N NaOH and samples were incubated in a shaking waterbath (95 rpm) at 37^{o}C for 2 h. Samples were centrifuged at 5,525 x g for 1 h and the supernatant (digestate) was isolated, aliquoted and stored at -20^{o}C. A digestate blank was prepared in the same manner but the 2 mL sample was substituted with 2 mL HBSS. Prior to addition to cells, all digestates were sterile filtered (0.22 µm filters, Fisher Scientific, Dublin, Ireland).

Cell culture

Jurkat T cells, a human leukemic T cell line, were maintained in antibiotic-free RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and plated at a density of 1 x 10^5 and 2 x 10^5 cells/mL for cell proliferation and ELISA assays, respectively. RAW 264.7 murine macrophages were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM) media supplemented with 10% FBS and were seeded at a density of 0.2 x 10^5 cells/mL for ELISA assays. Cells were maintained at 37^{o}C in a 5% CO_2 atmosphere. Reduced serum media (2.5% FBS) was used for all experiments.

Cell proliferation

The (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to measure the effect of the digestates on cell proliferation in Jurkat T cells and RAW 264.7 cells. Cells were incubated for 24 h at 37^{o}C with 0 - 10 % (v/v) of the supplemented milk digestates in a 96 well plate. The MTT assay kit (MTT I proliferation kit, Roche Diagnostics, West Sussex, UK) was then used to measure cell proliferation. Absorbance was determined at 570 nm using a microplate reader (VarioskanFlash, Thermo Scientific, Massachusetts, USA) and cell proliferation was calculated as a percentage of the control, untreated cells. Concentrations of 2.5 % (v/v) digestates were selected for analysis of potential anti-inflammatory effects based on the results of the MTT assay.

Cytokine production

Jurkat T cells were stimulated using 50 µg/mL Concanavalin-A (ConA) and incubated for 24 h at 37^{o}C with 2.5% (v/v) digestates. The production of cytokines interferon-γ (IFN-γ), interleukin-2 (IL-2) and interleukin-6 (IL-6) were measured by ELISA. RAW 264.7 cells were allowed to adhere overnight. Cells were incubated for a further 24 h at 37°C with 2.5% (v/v) digestates and 0.1 µg/mL LPS. The production of cytokines IL-6, interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) was measured by ELISA. Cytokine production was determined using eBioscience ELISA kits (Ready-SET-Go kit purchased from eBioscience, Hatfield, UK). Absorbance was measured at 450 nm on a microplate reader (VarioskanFlash, Thermo Scientific, Massachusetts, USA) and cytokine production was expressed as percentage of ConA-stimulated Jurkat T cells or LPS-stimulated RAW 264.7 cells.

Statistical analysis

All data represent the mean ± standard error (SE) of at least three independent experiments. Statistical analysis was by one-way analysis of variance (ANOVA) followed by Dunnett's test or Tukey's test as analysed using GraphPad Prism 4 (GraphPad software, California, U.S.A.).

Results and Discussion

Supplementation of low fat milk with BSG hydrolysates

The BSG Alcalase hydrolysate, U, and its associated membrane fractions were added to low-fat milk and subjected to SGID to determine if the supplemented milk products possess potential anti-inflammatory activity following digestion. The degree of hydrolysis (DH) of U was 12.1%, as measured using the trinitrobenzenosulfonic (TNBS) method (Connolly *et al*., 2015). Gel permeation chromatography revealed that the molecular mass distribution profile of U and its associated fractions was > 15 kDa (31.35 %), 15 - 5 kDa (~14 %), 5 - 1 kDa (~20%) and < 1 kDa (~35%). Ultrafiltration enriches permeates with low molecular weight proteins and the retentates contained higher molecular weight proteins (Connolly *et al*., 2015). The majority of peptides with antioxidant activity

have a lower molecular weight (500-1800Da) (Samaranayaka *et al*., 2011) whereas higher molecular weight peptides have previously demonstrated greater anti-inflammatory activity (McCarthy *et al*., 2013b).

Hydrolysates were added to low-fat milk at a concentration of 12.5 ppm which is a similar concentration to that used in previous studies (McCarthy *et al*., 2015). Milk and other dairy beverages are common vehicles for functional food ingredients including probiotics, omega-3 fatty acids, plant sterols, vitamins and minerals and bioactive peptides. Several dairy-based products containing added bioactive peptides with health or functional properties have been launched on the market (Dziuba and Dziuba, 2014).

Cytotoxicity of digested milk with added BSG hydrolysates

Jurkat T cells were incubated with BSG protein hydrolysate-supplemented milk digestates (1%-10%, v/v) for 24 h and cell proliferation was measured using the MTT assay. There was no decrease in cell proliferation observed up to a concentration of 2.5% (v/v) digestate for any of the samples investigated (Table 1). At the 5% and 10% (v/v) concentrations all of the digested milk samples caused a significant (P<0.05) decrease in cell viability (Table 1). The digestate blank was not cytotoxic at 10% (v/v) indicating that the digestive enzymes were not responsible for the cytotoxic effects of the digested milk samples (Table 1). A non-toxic concentration of 2.5% (v/v) digestate for all samples was selected for further experiments in Jurkat T cells. Digestate at 2.5% (v/v) was also non-toxic in RAW 264.7 cells (data not shown).

Immunomodulating effect of digested milk with added BSG hydrolysates

Two models of inflammation were investigated in the present study, a ConA-stimulated Jurkat T cell model and an LPS-stimulated mouse macrophage RAW 264.7 model. IL-6 production was significantly decreased (P<0.05) in stimulated Jurkat T cells in the presence of BSG protein hydrolysate-supplemented milk digestates (Table 2). Supplementation of milk with U >5 decreased IL-6 production to 79.4% of the ConA-stimulated control cells followed by U <3, U <5 and U which reduced IL-6 production to 82.3%, 83.5% and 85.1%, respectively.

Table 1: The effect of BSG protein hydrolysate-fortified milk simulated gastrointestinal digestates (1%-10% v/v) on cell proliferation in Jurkat T cells.

	Cell Viability (% of untreated control cells)				
	1% v/v digestate	2% v/v digestate	2.5% v/v digestate	5% v/v digestate	10% v/v digestate
Digestate blank	102.5 ± 4.7	120.0 ± 4.2	115.9 ± 9.0	115.2 ± 4.7	122.0 ± 3.9
Unfortified milk	108.2 ± 5.4	108.0 ± 2.7	104.3 ± 7.9	60.7 ± 15.2*	26.9 ± 21.9*
Milk + U	101.9 ± 3.9	102.8 ± 5.6	92.0 ± 8.0	62.9 ± 15.5*	15.0 ± 6.8*
Milk + U<3	95.7 ± 9.9	102.4 ± 11.0	85.9 ± 7.6	58.6 ± 17.3*	18.4 ± 13.2*
Milk + U<5	107.8 ± 8.4	106.1 ± 4.5	97.0 ± 6.4	63.1 ± 12.7*	20.9 ± 16.7*
Milk + U>5	95.2 ± 5.3	90.2 ± 6.3	83.4 ± 12.2	25.3 ± 9.1*	3.5 ± 1.2*

The values are expressed as a percentage relative to untreated Jurkat T cells, mean ± SE of five independent experiments. The digestate blank represents a digestion containing HBSS and digestive enzymes only. Unfortified milk represents a digestion of milk with no added BSG protein hydrolysate. U represents unfractionated BSG protein hydrolysate; U <3, <5 and >5 indicate fractionated BSG protein hydrolysate. * Denotes statistically significant difference in cell viability, relative to untreated Jurkat T cells (P<0.05), ANOVA followed by Dunnett's Test.

Table 2: The effect of BSG protein hydrolysate-fortified milk simulated gastrointestinal digestates (2.5 % v/v) on cytokine production in Concanavalin-A (ConA) stimulated Jurkat T cells.

Cytokine Production (% of control, ConA-stimulated cells)			
	IFN- γ	IL-6	IL-2
Digestate Blank	88.5 $\pm$ 12. 4	91.5 $\pm$ 4.1 $^{(U>5)}$	98.4 $\pm$ 2.4
Unfortified Milk	108.1 $\pm$ 9.7	91.6 $\pm$ 1.8 $^{(U>5)}$	98.2 $\pm$ 6.6
Milk + U	90.1 $\pm$ 7.4	85.1 $\pm$ 3.4*	96.0 $\pm$ 4.4
Milk + U<3	95.4 $\pm$ 4.5	82.3 $\pm$ 7.0*	95.1 $\pm$ 6.3
Milk + U<5	111.1 $\pm$ 18.8	83.5 $\pm$ 0.8*	94.2 $\pm$ 3.2
Milk + U>5	86.9 $\pm$ 4.9	79.4 $\pm$ 2.4* $^{(DB,UF)}$	91.9 $\pm$ 3.3

The values are expressed as a percentage relative to Jurkat T cells treated with ConA alone, mean ± SE of three independent experiments. The digestate blank represents a digestion containing HBSS and enzymes only. Unfortified milk represents a digestion of milk with no added BSG protein hydrolysate. U represents unfractionated BSG protein hydrolysate; U <3, <5 and >5 indicate fractionated BSG protein hydrolysate. * denotes statistically significant difference in cytokine production relative to control Jurkat T cells (P<0.05), $^{(DB)}$ denotes significant difference relative to digestate blank (P<0.05), $^{(UF)}$ denotes significant difference relative to unfortified milk (P<0.05), $^{(U>5)}$ denotes significant difference relative to milk + U>5 (P<0.05), ANOVA followed by Tukey's Test.

The secretion of IL-2 and IFN-γ was not altered in ConA-induced Jurkat T cells incubated with digested BSG hydrolysate-supplemented milk samples (Table 2). The BSG protein hydrolysate, U, and its 5kDa retentate (U >5), previously demonstrated anti-inflammatory effects at a concentration of 0.5% (w/v), when added directly to cells, by reducing the production of IFN-γ in ConA-stimulated Jurkat T cells (McCarthy *et al*., 2013b). The inability of SGID BSG-supplemented milk to reduce the secretion of IFN-γ in Jurkat T cells could result from the lower concentration of sample added to the cells as a consequence of their dilution in the milk and the simulated digestion fluid, or it could also indicate that the peptides responsible for the reduction in IFN-γ production were not stable to gastrointestinal enzymes. It has previously been observed that BSG hydrolysate, U, is relatively stable to SGID as ACE-inhibitory values were similar before and after digestion (Connolly *et al*., 2015) therefore, it seems likely that in order to elicit a reduction in IFN-γ the concentration of hydrolysate incorporated into the food will need to be increased. Increasing the hydrolysate content in milk may necessitate encapsulation; microencapsulation of whey protein concentrate hydrolysates (WPCH) by spray drying reduced the bitter taste and hydroscopicity of WPCH without altering the immunoregulatory activity (Ma *et al*., 2014) and may also provide a suitable method for supplementation of foods with BSG hydrolysates.

None of the digested milk samples significantly altered the production of IL-6, IL-1β or TNF-α in LPS-stimulated RAW 264.7 cells (Table 3) which may be indicative of a cell specific effect (Table 3). Hydrolysates from a variety of sources including salmon fin and almonds have demonstrated the ability to inhibit inflammatory cytokine release in RAW 264.7 cells (Ahn *et al*., 2015; Udenigwe *et al*., 2013). Again, it is possible that incorporating the hydrolysates into the milk at a higher concentration may improve the anti-inflammatory effects in RAW 264.7 cells.

The action of digestive enzymes can alter the chemical structure and bioactivity of food components including peptides (Wang *et al*., 2015). Several

Table 3: The effect of BSG protein hydrolysate-fortified milk simulated gastrointestinal digestates (2.5 % v/v) on cytokine production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells.

Cytokine Production (% of control LPS-stimulated cells)			
	IL-1β	IL-6	TNF-α
Digestate Blank	88.1 ± 9.2	114.7 ± 11.6	108.0 ± 14.8
Unfortified Milk	133.7 ± 12.4	122.6 ± 5.9	100.3 ± 13.7
Milk + U	114.1 ± 18.1	125.6 ± 13.6	86.0 ± 11.4
Milk + U<3	111.7 ± 13.6	108.3 ± 17.6	99.2 ± 1.2
Milk + U<5	126.3 ± 23.8	112.4 ± 8.5	111.7 ± 15.1
Milk + U>5	103.6 ± 13.0	110.5 ± 17.8	109.3 ± 10.6

The values are expressed as a percentage relative to RAW 264.7 cells treated with LPS alone, mean ± SE of three independent experiments. The digestate blank represents a digestion containing HBSS and enzymes only. Unfortified milk represents a digestion of milk with no added BSG protein hydrolysate. U represents unfractionated BSG protein hydrolysate; U <3, <5 and >5 indicate fractionated BSG protein hydrolysate with molecular weight <3, <5 and >5 kDa, respectively. Statistical analysis by ANOVA followed by Tukey's Test.

examples of the effect of digestion on the anti-inflammatory effects of food constituents may be cited. The anti-inflammatory activity of herbs and a natural eggshell membrane were found to be enhanced following *in vitro* digestion, as determined using a TNF-α-stimulated Caco-2 cell model and a mitogen-stimulated peripheral blood mononuclear cell model, respectively (Chohan *et al*., 2012; Benson *et al*., 2012). However, the anti-inflammatory activity of a fruit juice supplemented with pine bark extract was reduced in a cell model designed to replicate *in vivo* intestinal inflammation, following *in vitro* digestion (Frontela-Saseta *et al*., 2013).

The anti-inflammatory effects of an *in vitro* SGID snack-bar, chocolate-drink and yogurt with added BSG protein hydrolysates in Jurkat T cells have previously been reported (McCarthy *et al*., 2015). The findings indicated that the immunomodulatory effects of BSG protein hydrolysates may be influenced by the food vehicle in which they are incorporated. Digested samples containing a BSG protein hydrolysate, prepared using Flavourzyme, and its 5kDa retentate, 3kDa or 5kDa permeate caused a decrease in the production of IFN-γ in ConA-induced Jurkat T cells when a snack bar was used as the vehicle but had no effect on IFN-γ when incorporated into a sports drink or yogurt. Similarly, two of the fractions (<3kDa and <5kDa) enhanced the IL-2 reducing activity of digested yogurt in ConA-induced Jurkat T cells but had no effect when delivered in a snack bar or sports drink (McCarthy *et al*., 2015).

Milk proteins may be hydrolysed to produce bioactive peptides during gastrointestinal digestion (De Noni, 2008). However, no change was found in the production of cytokines in either the Jurkat T cell or RAW 264.7 cell model in the presence of digested, unsupplemented milk samples (Table 2 & 3).

Conclusions

It is important that nutraceuticals demonstrate an ability to retain their bioactivity following digestion. Several factors, such as the food matrix, can influence the anti-inflammatory activity of BSG protein hydrolysates following their incorporation into a food vehicle and subjection to gastrointestinal digestion. The immunomodulatory effects of the digested BSG hydrolysate supplemented milk appear to be cell specific and certain cytokines (e.g.: IL-6) may be more

susceptible to modulation. The results of our study indicate that BSG hydrolysates can confer some anti-inflammatory effects in Jurkat T cells following supplementation into milk and SGID. However, further research, including additional inflammatory markers, is necessary to optimise the anti-inflammatory effect of BSG hydrolysate supplemented food products.

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Chapter 3

Aqueous and enzyme-extracted phenolic compounds from brewers' spent grain (BSG): assessment of their antioxidant potential.

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Abstract

Brewers' spent grain (BSG) is a major co-product of the brewing industry and a potential valuable source of protein, cell wall polysaccharides, lignin, lipid and phenolic compounds. The aim of this study was to assess the antioxidant potential of phenolic extracts isolated from BSG using cell wall degrading enzymes, Depol 740L, Shearzyme and Ultraflo Max. The phenolic extracts were prepared from black BSG (derived from barley grains roasted at 200°C) and pale BSG (derived from malted barley grains). The phenolic extracts protected against hydrogen peroxide (H_2O_2)-induced DNA single strand breaks in U937 cells as assessed using the comet assay. The extracts also protected against a H_2O_2 challenge in HepG2 cells, as assessed by measuring the cellular content of glutathione (GSH) and the activity of cellular antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT). Enzyme-extracted black and pale BSG phenolic extracts protected against oxidant-induced DNA damage and enhanced the cellular antioxidant activity in cells.

Introduction

Brewers' spent grain (BSG) is a residue from the production of wort for the brewing industry and is comprised of the husk, pericarp and seed coat layers of barley (McCarthy *et al*., 2012). Currently, BSG is primarily used as an animal feed. However, BSG contains approximately 20g protein and 70g fibre per 100g (Mussatto *et al*., 2006) and is also a potentially valuable source of bioactive phytochemicals such as phenolic compounds (Connolly *et al*., 2013; McCarthy *et al*., 2014; Connolly *et al*., 2015). BSG derived from the standard brewing process is referred to as pale BSG. Black BSG, a product of dark wort production, is obtained following the roasting of barley grain at 200°C (Connolly *et al*., 2013; Piggott *et al*., 2014). The principal category of phenolic compounds identified in pale and black BSG are the hydroxycinnamic acids (HCAs) which are primarily comprised of *p*-coumaric acid, caffeic acid, ferulic acid and their derivatives (Teixeira *et al*., 2013). The total HCA content of pale and black BSG was previously shown to be 555.29 and 237.70 μg g^{-1} BSG dry weight (DW),

respectively (McCarthy *et al*., 2013) and ferulic acid was the predominant phenolic compound.

Oxidative stress is an imbalance between reactive oxygen species (ROS) production and the activity of the cellular antioxidant defences. HCAs have demonstrated antioxidant activity and research has shown that HCAs can be used as preventive and/or therapeutic agents in several diseases related to oxidative stress including atherosclerosis, inflammatory injury, cancer and cardiovascular diseases (Teixeira *et al*., 2013). Previous studies have demonstrated the significant antioxidant potential of phenolic extracts prepared from BSG using an alkaline extraction method (McCarthy *et al*., 2012; McCarthy *et al*., 2014; Piggott *et al*., 2014). However, alkaline extraction is a crude procedure which has been shown to result in the degradation of phenolic acids and also increases the salt content of the extracts (Krygier *et al*., 1982; Nardini *et al*., 2002). Enzymatic treatment, using carbohydrases, has also been investigated as it has the added benefit of facilitating the release of bioactives which may be bound to the cell wall (Alrahmany and Tsopmo, 2012). Enzymatic hydrolysis is a potential extraction method for BSG components due to the specificity and mild reaction conditions required (Forssell *et al*., 2008).

The aim of this research was to assess the *in vitro* antioxidant activity of BSG extracts prepared using the carbohydrase Depol 740L or a combination of Depol 740L, Shearzyme and Ultraflo Max. Depol 740L is a feruloyl esterase which has been shown to be effective in the release of bound ferulic and *p*-coumaric acid (Forssell *et al*., 2008). Shearzyme 500L is a xylanase and Ultraflo Max is a β-glucanase–xylanase mixture (Hu *et al*., 2014). Extracts were prepared from wet (75% moisture) and dry samples (11% moisture) of both pale and black BSG. The DNA protective effects of BSG phenolic extracts were measured by their ability to protect against hydrogen peroxide (H_2O_2)-induced DNA single strand breaks in U937 cells using the comet assay. The impact of BSG phenolic extracts on the cellular antioxidant status in HepG2 was assessed by measuring the cellular content of glutathione (GSH) and the activity of the enzymes superoxide dismutase (SOD) and catalase (CAT) following an oxidative challenge.

Materials and Methods

Materials

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich Ireland Limited, (Co. Wicklow, Ireland). HepG2 cells and U937 cells were obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). Shearzyme 500 L (declared activity 500 Fxu g^{-1}) and Ultraflo Max (declared activity 470 Fxu g^{-1}) were purchased from Novozymes A/S, Bagsværd, Denmark. Depol 740L (declared activity 945 u g^{-1}) was purchased from Biocatalysts Ltd., Cardiff, UK.

Preparation of Phenolic Extracts

Whole BSG samples were obtained from the same brewery. For dry BSG, samples were oven-dried for 18 hours, at 60°C. Both pale and black BSG were stored in 1 kg quantities in polyethylene bags which were vacuum-packed and maintained at -20°C. To generate phenolic extracts, BSG was sheared in distilled H_2O (1:10) at 24,000 rpm for 2 minutes using an Ultra Turrax®T25 high-performance disperser (IKA® Werke GmbH & Co. KG, Staufen, Germany). The BSG suspension was then incubated with carbohydrases: Shearzyme 500 L, Ultraflo Max and/or Depol 740L for either 240 or 540 minutes, at 50°C and pH 5 or pH 7 (Table 1). The carbohydrase enzymes were heat deactivated at 80°C for 20 minutes in a waterbath. Following centrifugation at 2,700 g for 20 minutes at 10°C (Hettich Zentrifugen Universal 320R centrifuge, Andreas Heittich GmbH & Co., Tuttlingen, Germany), the supernatant was retained and stored at -20°C prior to analysis. Phenolic preparations from black, wet BSG were designated BW1-4; from black, dry BSG were designated BD1-2; from pale, wet BSG were designated PW1-5 and from pale, dry BSG were designated PD1-3, as detailed in Table 1. Samples BW1 and PW1 were obtained by shearing BSG in distilled H_2O without the addition of carbohydrase enzymes. These samples were used as control samples throughout the experiments with BW1 being used as a control for all black BSG extracts and PW1 being used as a control for all pale BSG extracts. The samples investigated were selected based on a pre-screening process which

Table 1: Extraction procedure used for the generation of BSG phenolic extracts.

Sample code	BSG type	Enzyme	Enzyme volume (µL g^{-1} BSG_{dw})	pH	Extraction time (minutes)
BW1	Black Wet		0	7	240
BW2	Black Wet	Depol 740L	40	7	240
BW3	Black Wet	Depol 740L	60	7	240
BW4	Black Wet	Shearzyme/Ultraflo Max/Depol 740L	60 (each)	7	240
BD1	Black Dry	Depol 740L	30	5	240
BD2	Black Dry	Depol 740L	60	5	240
PW1	Pale Wet		0	7	240
PW2	Pale Wet	Depol 740L	100	5	240
PW3	Pale Wet	Depol 740L	100	7	240
PW4	Pale Wet	Shearzyme/Ultraflo Max	75 (each)	5	240
PW5	Pale Wet	Shearzyme, Ultraflo Max/ Depol 740L	75 (each)	5	240
PD1	Pale Dry	Depol 740L	10	5	240
PD2	Pale Dry	Depol 740L	100	7	240
PD3	Pale Dry	Shearzyme, Ultraflo Max/ Depol 740L	100 (each)	5	540

Black BSG was derived from barley grains roasted at 200°C and pale BSG was derived from malted barley grains. Wet BSG contains < 75% moisture (Connolly *et al*., 2013). Dry BSG contains <10% moisture (Santos *et al*., 2003). All samples were treated with enzymes at 50°C.

identified the most active fractions as analysed by Ferric reducing antioxidant power (FRAP); 2,2-Diphenyl-1-picrylhydrazyl (DPPH•); Oxygen radical absorbance capacity (ORAC); 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS+); Dipeptidyl peptidase-4 (DPP4) and angiotensin-converting-enzyme (ACE) inhibition.

Total Phenolic Content (TPC)

The Folin–Ciocalteu assay, as described previously (Singleton and Rossi, 1965), was used to measure the TPC of BSG phenolic extracts. This assay is based on the capacity of a compound to reduce the Folin–Ciocalteu reagent (FCR), and thus the TPC method is non-specific to phenols and measures the presence of any reducing agent. Following reaction of the samples with FCR, the absorbance was measured at 765 nm relative to a distilled H_2O blank. The phenolic content, expressed as gallic acid equivalents (GAE), was determined using gallic acid as a standard. Results were expressed as milligram gallic acid equivalents per mL phenolic extract (mg GAE mL^{-1}).

DPPH• Assay

The DPPH• radical scavenging potential of the extracts was performed according to the method described previously (Brand-Williams *et al*., 1995). The assay allows a measure of antioxidant potential, based on the ability of a compound to scavenge the DPPH radical by hydrogen donation. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2- carboxylic acid), ranging from 0.04 to 0.40 µM was used to prepare a standard curve. Absorbance values were determined at 515 nm, following a 30 min incubation with the DPPH reagent (0.0238 µg/ml). Results were expressed as %DPPH• inhibition: %DPPH• inhibition = $[(Abs_{Blank} - Abs_{Sample})/Abs_{Blank}] \times 100$.

FRAP Assay

The FRAP assay (Benzie and Strain, 1996), was used to determine to ability of extracts to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}). The reducing power of extracts was measured spectrophotometrically at 593 nm. Ferrous sulphate heptahydrate (0–100 µM) was used to prepare a standard curve and results were expressed as µM FRAP.

Cell Culture

HepG2 cells, a human liver carcinoma cell line, were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and plated at a density of 0.5×10^5 and 2×10^5 cells mL^{-1} for cell viability and cellular antioxidant assays, respectively. U937 cells, a human leukemic monocytic lymphoma cell line, were maintained in antibiotic-free RPMI-1640 medium supplemented with 10% (v/v) FBS and plated at a density of 2×10^5 cells mL^{-1} and 1×10^5 cells mL^{-1} for cell viability and comet assays respectively. Cells were maintained at 37°C in a 5% CO_2 atmosphere. Reduced serum media (2.5% FBS) was used for all experiments.

Cell Viability

The (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to measure the effect of the extracts on cell viability in HepG2 cells and U937 cells. Cells were incubated for 24 hours at 37°C with BSG extracts at concentrations of 0 to 20% v/v of the extracts. The MTT assay kit (MTT I proliferation kit, Roche Diagnostics, West Sussex, UK) was then used to measure cell viability. Absorbance was determined at 570 nm using a microplate reader (VarioskanFlash, Thermo Scientific, Waltham, MA) and cell viability was calculated as a percentage of the control, untreated cells. An inhibitory concentration-50 (IC_{50}) value, the concentration of a compound that reduces cell viability to 50% of the untreated control cells, was determined for each of the phenolic extracts.

Comet Assay

U937 cells were seeded in a 12-well plate at a density 1 x 10^5 cells mL^{-1} in RPMI-1640 medium with 2.5% FBS. Cells were incubated for 24 hours with either BSG phenolic extracts BW1-4 and BD1-2 (0.1% v/v) or PW1-5 and PD1-3 (4% v/v) or ferulic acid (1 μg mL^{-1}). Cells were subsequently treated with H_2O_2 (75 μM for 30 minutes). Oxidative DNA damage to the U937 cells was then assessed using the comet assay adapted from Tice *et al*. (1991). Fifty cells were scored for each slide using a fluorescence microscope (Optiphot-2, Nikon) and the Komet 5.5 image analysis software. Data were expressed as percent tail DNA.

Cellular Antioxidant Assays: Glutathione, Superoxide Dismutase and Catalase

HepG2 cells (2 x 10^5 cells mL^{-1}) were incubated with BSG phenolic extracts BW1-4 and BD1-2 (0.5% v/v) or PW1-5 and PD1-3 (4% v/v) for 24 hours at 37°C. Following incubation, cells were exposed to 2 mM H_2O_2 for 120 minutes. Cells were harvested, sonicated and centrifuged (800 rpm, 10 minutes) and the supernatant was collected for the determination of antioxidant enzyme activity.

The glutathione (GSH) content of the cells was measured according to the method described previously (Hissin and Hilf, 1976). The GSH content was measured in cells as an indicator of intracellular non-enzymatic antioxidant defences. Briefly, 100 μL supernatant was mixed with sodium phosphate–ethylenediaminetetraacetic acid buffer (1.8 mL) and *σ*-phthaldialdehyde (0.1 mg). The fluorescence intensity of the samples was determined (Thermo Scientific Varioskan Flash microplate reader) at wavelengths of 360 nm (absorption) and 430 nm (emission). The GSH content of the samples was determined from a standard curve (0–2.5 nmol mL^{-1} GSH).

The activity of cellular superoxide dismutase (SOD) was determined using the method previously described by Misra and Fridovich, (1977). Briefly, the cell supernatant was diluted in 0.05 M potassium phosphate buffer (pH 7) and xanthine, xanthine oxidase and cytochrome c were added. The xanthine oxidase system generates superoxide anion which reduces cytochrome c and this reaction is inhibited by SOD. The reduction in cytochrome c was used to determine the

activity of SOD present in the samples from a standard curve (0-1µg SOD mL^{-1}). Samples were read at 550 nm at 20 minute intervals for at least 5 readings.

Catalase (CAT) activity was determined using a modification of the method of Baudhuin *et al*. (1964). H_2O_2 was added to the cell supernatants and following incubation any remaining H_2O_2 was determined spectrophotometrically at 465 nm. One unit of catalase activity was defined as the amount of catalase required to decompose 1 µmol H_2O_2 per min at pH 7.5 and 25°C.

GSH content and SOD and CAT activity were quantified relative to the protein content as nmol mL^{-1} GSH mg^{-1} protein in cell homogenate and SOD and CAT units mg^{-1} protein, respectively. The protein content of the samples was quantified by the bicinchoninic acid (BCA) protein assay as previously described (Smith *et al*., 1985). Data were expressed as a percentage of untreated control cells.

Statistical Analysis

All data represent the mean ± standard error (SE) of at least three independent experiments. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett's test as analysed using GraphPad Prism 4 (GraphPad software, La Jolla, CA).

Results and discussion

Alkaline extracts derived from BSG have been shown to protect against oxidant-induced DNA damage and oxidant-induced depletion of cellular antioxidant markers (McCarthy *et al*., 2012; McCarthy *et al*., 2014). Acid and alkaline hydrolysis are the most common extraction methods for phenolic compounds but may cause degradation of these compounds. The use of enzymatic hydrolysis is an alternative method, whereby enzymes are used to degrade carbohydrate linkages and release bound phenolics (Stalikas, 2007). The aim of the present research was to assess the DNA protective effects and cellular antioxidant potential of phenolic-rich extracts obtained from BSG using an enzyme-aided extraction procedure. BSG may be subjected to various drying techniques such as oven

drying and freeze-drying, to increase its use and marketability (Santos *et al*., 2003). The antioxidant activity of extracts prepared from both wet and dry (75% and 11% moisture, respectively) black and pale BSG were compared.

Antioxidant Power of BSG Phenolic Extracts

The TPC content of the BSG extracts prepared using different carbohydrase combinations ranged from 0.015 to 0.056 mg GAE mL^{-1} (Table 2) and in general, the average TPC was lower in the pale BSG extracts in comparison to the black BSG extracts. Similarly, in a study of alkaline-extracted BSG phenolic extracts, the black phenolic extracts also had a higher average TPC compared with pale phenolic extracts (McCarthy *et al*., 2012). It has previously been shown that roasting barley grain increases the TPC, proportionate to roasting time, owing to the formation of Maillard browning reaction products (MRP) (Omwamba and Hu, 2010; Dabina-Bicka *et al*., 2011), also resulting in the simultaneous formation of compounds with antioxidant properties (Piggott *et al*., 2014). The TPC method is not specific to phenolic compounds as it measures any compound which is capable of reducing Folin–Ciocalteu reagent including MRP (Huang *et al*., 2005).

The BSG extracts prepared using alkaline extraction (McCarthy *et al*., 2012) had higher TPC than the carbohydrase extracts (Table 2). Chemical extractions achieve high quantities of released products (Tang *et al*., 2016), however, these procedures involve high temperature and harsh acidic and alkaline conditions which could destroy other components. Enzymatic fractionation methods on the other hand take advantage of enzymes' high selectivity and thus allow more targeted and effective fractionation processes. Therefore, it is not surprising that values obtained from our earlier studies (McCarthy *et al*., 2012) using alkaline extraction were higher than reported in the present study. There were no statistically significant differences across any of the values for TPC (Table 2). Therefore, neither increasing enzyme concentration, the drying of the samples or alteration of the pH during extraction had a significant effect on the quantity of phenolics released from the samples.

Table 2: Total phenolic content (TPC), 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical scavenging activity and ferric reducing antioxidant power (FRAP) of BSG phenolic extracts.

Sample Code	TPC (mg GAE mL^{-1})	DPPH• scavenging (%)	FRAP (μM)
BW1	0.048 ± 0.006	57.29 ± 12.43	148.62 ± 5.16
BW2	0.043 ± 0.003	42.47 ± 5.46	136.27 ± 0.90
BW3	0.032 ± 0.002	42.12 ± 13.44	140.80 ± 1.64
BW4	0.056 ± 0.002	59.23 ± 12.63	146.68 ± 7.02
BD1	0.023 ± 0.003	22.64 ± 13.64	126.22 ± 9.66
BD2	0.052 ± 0.012	54.86 ± 16.68	135.06 ± 10.01
PW1	0.015 ± 0.001	37.99 ± 14.21	114.59 ± 0.57
PW2	0.037 ± 0.008	24.18 ± 8.95	117.10 ± 7.13
PW3	0.016 ± 0.006	12.89 ± 1.93	114.39 ± 5.77
PW4	0.030 ± 0.01	23.62 ± 5.48	116.25 ± 6.73
PW5	0.037 ± 0.009	28.82 ± 7.78	117.51 ± 5.90
PD1	0.024 ± 0.006	25.80 ± 4.15	114.15 ± 5.65
PD2	0.024 ± 0.005	18.71 ± 2.76	114.91 ± 6.60
PD3	0.032 ± 0.007	20.24 ± 5.76	115.66 ± 6.40

Values are mean ± SE of three independent experiments. BW1-4 represent extracts from black, wet BSG; BD1-2 represents extracts from black, dry BSG; PW1-5 represents extracts from pale, wet BSG and PD1-3 represents extracts from pale, dry BSG.

The radical scavenging ability (DPPH•) of BSG extracts (Table 2) ranged from 12.89-59.23%. DPPH• scavenging activity of black BSG extracts showed a similar trend to the TPC values. With the exception of BD1, black BSG extracts demonstrated higher DPPH• scavenging activities compared with pale BSG extracts. Similarly, McCarthy *et al.* (2013) showed that alkaline-extracted black BSG extracts had a higher average DPPH• radical scavenging ability compared with their pale counterparts. Papetti *et al.* (2006) showed that roasted barley had a higher DPPH• scavenging ability compared with natural barley. There was no significant difference in the DPPH• activity of any of the samples tested regardless of the extraction procedure used.

Black BSG extracts had a higher average ferric reducing power compared to pale BSG extracts. A similar trend was noted by McCarthy *et al.* (2012), where alkaline-extracted black BSG extracts showed a higher average FRAP activity compared to pale extracts. Similarly, Piggott *et al.* (2014) showed that alkaline extracted black BSG phenolics demonstrated higher FRAP activity compared with the corresponding pale BSG phenolic extracts. In the present study, there was no statistically significant differences across the FRAP values between all the samples.

Overall however, there was a reasonable correlation between TPC and the other two antioxidant measurements, DPPH• and FRAP.

Cell Viability

Phenolic extracts from black BSG had a greater cytotoxic effect in the U937 cell line, compared to pale BSG extracts, as demonstrated by the lower IC_{50} values. IC_{50} values in U937 cells ranged from 0.7 to 9.6% (v/v) for black phenolic extracts and 19.1 to 45.0% (v/v) for pale phenolic extracts (Table 3). Most phenolic extracts from black BSG (BW1-4, BD2) demonstrated significant levels of cytotoxicity at concentrations above 1% (v/v) (Table 3), while some phenolic extracts from pale BSG (PW1 and PW4) did not demonstrate any significant cytotoxic effects within the concentration range tested (0-20% v/v) (Table 3).

Table 3: Effects of brewers spent grain (BSG) phenolic extracts (0-20% (v/v)) on cell viability in U937 cells.

	0.5%	1%	2%	2.5%	4%	6%	8%	10%	15%	20%	IC_{50} (% v/v)
BW1	58.6±4.1*	54.8±5.8*	49.8±6.9*	46.9±7.0*	40.8±6.5*	35.1±12.0*	32.0±7.1*	14.4±6.6*	10.6±5.5*	7.2±5.7*	1.5
BW2	84.8±9.9	66.2±2.7*	68.8±5.9*	57.1±4.8*	47.4±5.6*	48.6±7.5*	38.4±10.2*	28.7±4.8*	9.3±6.0*	1.6±1.6*	3.7
BW3	51.8±4.1*	46.1±3.5*	44.9±6.1*	42.6±4.5*	42.3±11.0*	36.4±5.9*	36.2±10.8*	21.0±4.1*	19.3±3.5*	18.0±4.9*	0.7
BW4	59.2±3.8*	53.0±4.9*	52.9±4.8*	53.9±7.7*	42.4±7.6*	56.3±9.2*	45.2±7.1*	23.7±6.3*	20.8±1.7*	0.0±0.0*	2.2
BD1	71.9±8.6	72.4±7.4	70.8±6.3	57.0±14.3*	69.2±12.2	68.8±12.3	64.3±14.7	43.8±5.8*	25.2±14.7*	23.6±12.9*	9.6
BD2	80.1±10.5	64.6±4.7*	65.7±5.6*	66.7±3.8*	66.5±3.6*	53.1±7.0*	51.5±11.7*	45.6±8.8*	41.1±8.6*	28.8±4.5*	7.8
PW1	105.7±10.4	114.9±9.7	105.2±9.0	109.5±11.9	103.6±12.9	100.5±16.0	99.3±11.1	94.7±12.4	90.2±9.5	73.0±6.7	26.0
PW2	122.9±5.2	115.5±8.6	117.5±3.9	123.6±9.6	107.7±0.5	113.2±3.7	98.8±1.9	101.8±8.2	75.6±2.5*	61.1±7.2*	21.8
PW3	107.1±1.9	105.6±3.2	94.4±4.1	99.8±3.8	91.5±6.2	86.4±1.6	73.6±4.6*	69.4±0.8*	60.7±1.2*	53.8±8.2*	20.3
PW4	120.9±12.5	124.3±13.0	110.3±9.1	111.5±14.5	99.1±15.1	91.8±14.2	90.4±13.8	89.1±13.7	79.6±12.2	56.1±6.0	22.7
PW5	107.1±7.8	115.1±4.0	111.5±11.1	105.1±17.0	104.8±15.8	88.0±17.0	94.6±13.1	80.5±6.6	64.7±0.3	48.1±2.5*	19.1
PD1	105.5±5.2	100.7±5.4	97.2±7.4	90.8±3.8	92.0±10.7	80.9±14.6	74.5±11.4	64.9±12.2	68.4±7.1	56.1±7.5*	23.9
PD2	108.2±3.7	101.3±2.8	94.8±3.0	90.5±4.5	89.9±3.4	79.9±0.8*	73.6±3.5*	64.2±2.5*	57.7±4.6*	53.3±2.1*	19.6
PD3	93.7±8.7	85.2±7.9	90.9±9.0	91.6±6.6	86.1±7.7	88.8±8.9	82.8±8.8	73.9±6.0	70.6±2.9	60.7±2.8*	45.0

Values are mean ± SE of three independent experiments, expressed as a percentage relative to untreated U937 cells. BW1-4 represent extracts from black, wet BSG; BD1-2 represents extracts from black, dry BSG; PW1-5 represents extracts from pale, wet BSG and PD1-3 represents extracts from pale, dry BSG. Statistical analysis by ANOVA followed by Dunnett's Test. * Denotes statistically significant difference in cell viability, relative to untreated U937 cells ($P<0.05$).

Previous research on alkaline-extracted BSG phenolic extracts found that black BSG extracts were more cytotoxic than their pale equivalents in U937 cells (McCarthy *et al*., 2014), which may be owing to the presence of MRPs generated during the roasting of barley. With the exception of extract BW3, BW1 had the lowest IC_{50} value, therefore it does not appear that the use of enzymes in the extraction procedure increases the cytotoxicity of the black BSG phenolic extracts (Table 3).

BSG phenolic extracts were less cytotoxic in HepG2 cells compared with U937 cells (Table 4). In HepG2 cells, black phenolic extracts, with the exception of extracts BW4 and BD2, demonstrated cytotoxic effects at concentrations exceeding 4% (v/v). The IC_{50} values of enzyme-treated black phenolic extracts was higher than that of BW1, suggesting that the carbohydrases were not toxic to HepG2 cells. Pale phenolic extracts, apart from extract PW3, did not demonstrate any significant toxic effects in HepG2 cells (Table 4). HepG2 cells possess a number of metabolising enzymes which facilitate the inactivation/detoxification of harmful substances (Knasmuller *et al*., 1998) and may therefore possess greater resistance to the cytotoxic effect of the BSG extracts in comparison to the U937 cells. Based on these results, non-toxic concentrations of 0.1% (v/v) for black extracts and 4% (v/v) for pale extracts were chosen for further analysis in U937 cells and concentrations of 0.5% (v/v) and 4% (v/v) for black and pale extracts respectively were selected for cellular antioxidant assays in HepG2 cells. This experimental approach was similar to that used by Karnjanapratum *et al*. (2016) to assess the antioxidant potential of gelatin hydrolysates from unicorn leatherjacket skin, utilising U937 cells to assess DNA protective effects and HepG2 cells to assess cellular antioxidant potential. Since U937 cells are suspension cells, the background DNA damage in control cells will be significantly lower than in adherent cells (HepG2 cells) which require scraping for analysis.

Comet Assay

To assess the DNA protective effects of the phenolic extracts from black and pale BSG, U937 cells were pre-incubated with the extracts for 24 hours prior to treating the cells with 75 μM H_2O_2 for 30 minutes. Exposure to H_2O_2 increased

Table 4: Effects of brewers' spent grain (BSG) phenolic extracts (0-20% (v/v)) on cell viability in the HepG2 cell line.

	0.5	1	2	2.5	4	6	8	10	15	20	IC_{50} (% v/v)
BW1	79.3±7.5	72.6±12.7	63.0±8.2	64.9±9.0	52.5±10.2*	57.5±10.2*	56.3±12.3*	56.5±7.7*	57.2±14.1*	52.0±6.7*	19.8
BW2	68.0±7.9*	61.7±1.7*	66.0±2.5*	58.3±7.1*	61.9±3.2*	56.4±5.0*	58.9±4.2*	50.8±7.0*	51.3±7.3*	47.0±6.9*	21.1
BW3	71.2±10.9	64.8±10.3	47.8±9.6*	67.3±9.1	57.3±4.8*	59.3±11.6*	46.9±8.2*	57.0±13.1*	46.1±9.9*	53.5±6.5*	39.0
BW4	72.0±11.7	79.5±23.2	61.9±8.7	61.3±15.1	57.8±14.2	52.1±14.2	56.1±16.2	56.4±16.9	55.2±15.7	54.0±10.8	22.0
BD1	83.5±6.4	85.6±4.5	78.8±4.4*	76.3±6.2*	78.9±7.1*	73.0±2.8*	70.9±1.7*	68.7±6.3*	66.1±3.3*	51.6±4.0*	37.6
BD2	93.7±12.3	92.6±18.0	87.4±7.3	77.2±11.6	84.9±13.3	74.7±9.8	77.8±7.8	70.4±10.1	70.1±10.7	67.6±14.4	78.4
PW1	103.1±3.2	94.5±4.7	98.1±3.3	100.6±5.4	109.6±8.2	109.2±12.1	103.8±9.2	99.0±5.6	105.5±10.7	97.6±8.7	n/d
PW2	114.3±2.5	115.8±9.8	109.6±3.7	119.8±13.1	109.0±14.2	120.5±19.8	122.0±17.5	127.1±20.5	127.8±18.2	115.8±17.3	n/d
PW3	63.7±2.5*	79.9±8.5	61.4±3.4*	58.6±3.2*	61.7±7.7*	62.1±5.5*	59.3±6.7*	59.7±3.4*	51.6±5.5*	39.1±15.9*	17.9
PW4	109.5±5.2	102.8±8.0	97.1±8.1	115.9±2.3	114.7±4.5	118.0±2.5	116.9±7.2	115.2±4.2	115.3±19.1	126.8±10.5	n/d
PW5	110.2±2.9	108.1±1.7	108.0±6.3	103.3±7.8	109.8±5.9	103.9±14.3	116.5±4.2	107.2±6.6	129.7±13.1	114.4±3.9	n/d
PD1	92.5±9.4	87.0±6.0	82.5±8.9	89.2±4.0	80.5±6.7	86.9±3.3	87.5±4.4	88.8±5.1	95.5±11.3	96.0±2.6	n/d
PD2	118.0±15.2	105.9±15.2	89.3±8.8	103.0±14.0	103.3±10.4	104.7±12.2	103.0±6.4	108.8±8.0	102.4±2.2	111.7±9.6	n/d
PD3	114.1±17.2	114.7±15.1	104.8±16.3	104.4±14.3	100.3±13.6	112.5±16.9	100.2±8.2	100.5±18.7	111.5±13.2	107.8±11.2	n/d

Values are mean ± SE of three independent experiments, expressed as a percentage relative to untreated HepG2 cells. BW1-4 represent extracts from black, wet BSG; BD1-2 represents extracts from black, dry BSG; PW1-5 represents extracts from pale, wet BSG and PD1-3 represents extracts from pale, dry BSG. Statistical analysis by ANOVA followed by Dunnett's Test. * Denotes statistically significant difference in cell viability, relative to untreated HepG2 cells ($P<0.05$). n/d: IC_{50} value could not be determined in the range of concentrations tested.

DNA damage from 6.1% to 62.5% tail DNA (Figure 1). Both black (0.1% v/v) and pale (4% v/v) BSG phenolic extracts (except PW3) significantly reduced H_2O_2-induced DNA damage in U937 cells (Figure 1). Extract BW4 significantly reduced tail DNA to 24.7% which was similar to the level of protection afforded by the positive control, 1 μg mL^{-1} ferulic acid which reduced DNA damage to 27.9% (Figure 1). In the pale BSG extracts, PW2 demonstrated the greatest protective effect and reduced DNA damage in H_2O_2 exposed U937 cells to 28.5% (Figure 1). No clear correlation was found between the TPC content and the DNA protective effects of the extracts however, extract PW3 had the least DNA protective effect and also the lowest TPC content (0.016 mg GAE mL^{-1}) and the highest DNA protective effect was observed for sample BW4 which had the highest TPC content (0.056 mg GAE mL^{-1}). The extracts produced without the use of enzymes (BW1 and PW1) were also effective in the protection against DNA damage (Figure 1).

H_2O_2 forms a hydroxyl radical in the presence of transition metal ions (Aruoma, 1998) which reacts with DNA bases or the deoxyribosyl backbone of DNA causing base damage and strand breaks (Valko *et al*., 2006). The direct quenching of reactive oxygen species (ROS), chelation of metal ions and regeneration of membrane-bound antioxidants by phenolic compounds have been proposed as mechanisms for their antioxidant action (Ji *et al*., 2015). Ferulic acid has previously demonstrated an ability to reduce H_2O_2-induced DNA breakage in HT-29 cells; (Ferguson *et al*., 2005) and ferulic, sinapic and caffeic acids have also shown a protective effect against 2,2'-azobis(2-amidinopropane dihydrochloride) (AAPH)-initiated oxidative damage in plasmid DNA (Shang *et al*., 2015). Similarly, Li *et al*. (2015) demonstrated that caffeic acid decreased H_2O_2-induced DNA double strand breaks in human embryo liver (L-02) cells. Evidence of the presence of both ferulic acid and caffeic acid in BSG has previously been shown (McCarthy *et al*., 2013).

Black BSG phenolic extracts at 0.1% (v/v) demonstrated a similar DNA protective effect to pale BSG phenolic extracts which were assessed at the higher concentration of 4% (v/v). Previous research on the DNA protective effects of alkaline-extracted BSG phenolic extracts noted that black extracts demonstrated greater antioxidant activity compared to pale extracts in H_2O_2-treated U937 cells

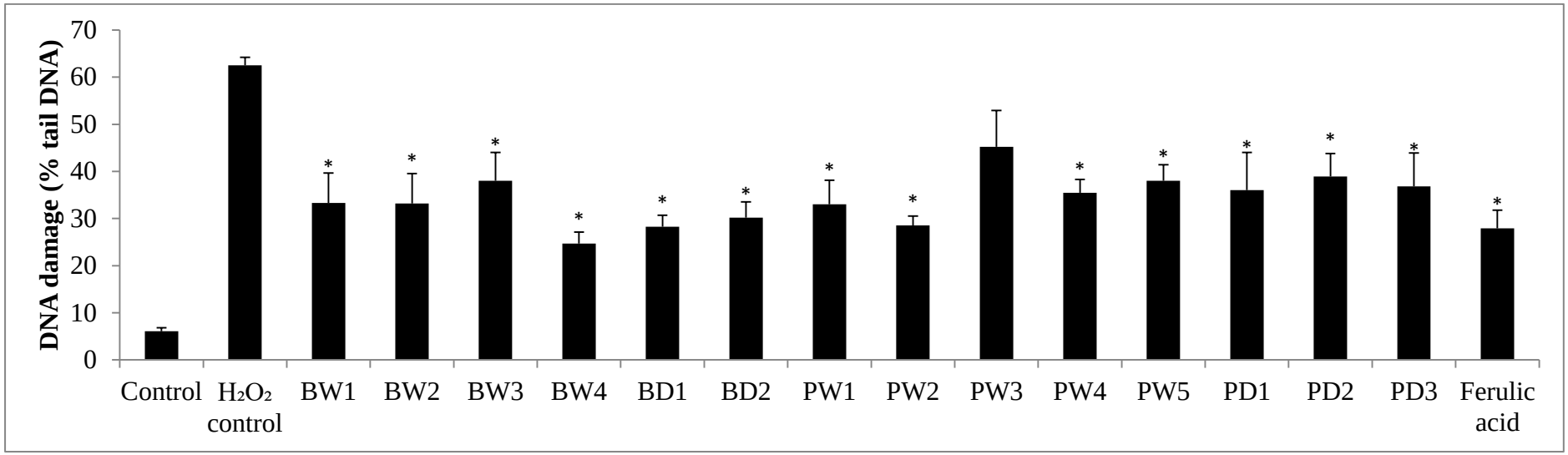


Figure 1: DNA damage in U937 cells treated with 75 μM H_2O_2 for 30 minutes following 24 hour incubation with black BSG extracts (0.1% v/v), pale BSG extracts (4% v/v) or ferulic acid (1 μg mL^{-1}). BW1-4 represent extracts from black, wet BSG; BD1-2 represents extracts from black, dry BSG; PW1-5 represents extracts from pale, wet BSG and PD1-3 represents extracts from pale, dry BSG. Values are mean ± SE of at least three independent experiments. Statistical analysis was by ANOVA followed by Dunnett's test. * Denotes significant difference ($P<0.05$) in DNA damage relative to H_2O_2 control.

(McCarthy *et al*., 2012). The higher total phenolic content in black BSG could be responsible for greater antioxidant activity of these extracts however; other non-phenolic compounds such as MRPs may also contribute to the antioxidant effects of the black BSG extracts. Wijewickreme and Kitts (1998) showed that direct incubation of PM2 bacteriophage DNA with brewed, boiled, or instant coffee MRPs, together with Fe^{2+}, protected against DNA breakage. It should also be noted however that certain individual compounds such as acrylamide produced during the heat treatment of foods may have genotoxic effects (Somoza, 2005). Therefore it would seem prudent to further characterise black BSG extracts to fully elucidate their effects.

Cellular Antioxidant Assays

Alkaline-extracted BSG phenolic extracts have previously demonstrated antioxidant effects in U937 cells by preventing H_2O_2-induced decreases in antioxidant enzyme activity (McCarthy *et al*., 2014). In the present study, the cellular antioxidant assays GSH, SOD and CAT were performed in oxidant-challenged HepG2 cells which were pre-incubated with BSG extracts. HepG2 cells were then challenged with 2mM H_2O_2 for 2 hours. A similar oxidant concentration and exposure time was used by Karnjanapratum *et al.* (2016); this is the standard experimental procedure adopted in our research group following previous investigation. HepG2 contain a large quantity of metabolising enzymes which are involved in the inactivation and detoxification of harmful substances (Knasmuller et al., 1998). Therefore higher concentrations of H_2O_2 were used in the HepG2 cell model (compared with the U937 model) in order to elicit an effect. With the exception of extract BW1 all of the extracts demonstrated significant (P<0.05) antioxidant activity in at least one cellular antioxidant enzyme assay. Treatment of HepG2 cells with H_2O_2 led to a significant reduction in GSH content (66.9%) compared to the untreated control (Figure 2A). Black BSG extracts BW2, BW3, BD1 and BD2 at a concentration of 0.5% (v/v) significantly (P<0.05) protected against a H_2O_2-induced decrease in GSH content, BW1 (extracted in the absence of enzymes) as well as BW4 did not significantly protect against GSH depletion. Pale phenolic extracts PD1, PD2, PD3, PW1, PW2, PW4 and PW5 at

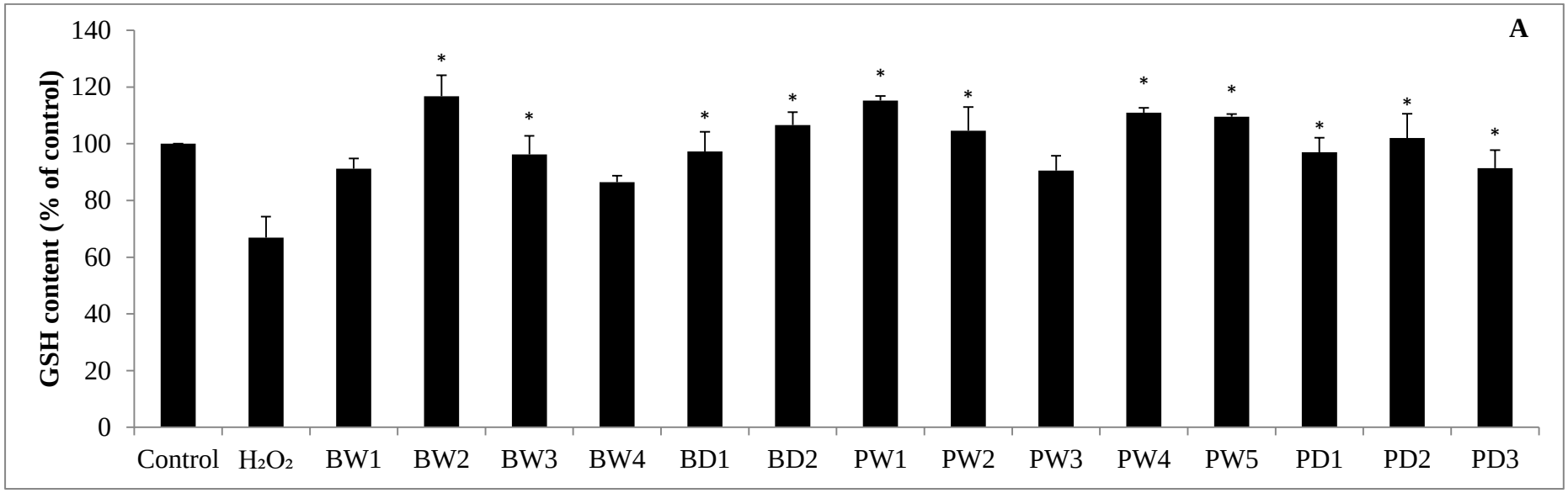


Figure 2A: Antioxidant potential of BSG black [0.5% (v/v)] and pale [4% (v/v)] phenolic extracts as assessed by their ability to protect cellular glutathione (GSH) content against H_2O_2-induced oxidative stress in HepG2 cells. BW1-4 represent extracts from black, wet BSG; BD1-2 represents extracts from black, dry BSG; PW1-5 represents extracts from pale, wet BSG and PD1-3 represents extracts from pale, dry BSG. Values represent the mean ± SE of at least three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * Statistically significant difference between sample and H_2O_2 control ($P<0.05$).

4% (v/v) demonstrated an antioxidant effect by protecting against H_2O_2-induced GSH depletion. In cells exposed to the pale extracts, the highest GSH content was found in the HepG2 cells treated with PW1 (extracted in the absence of enzymes) therefore, the use of enzymes in the extraction procedure does not appear to enhance the ability of the resulting pale BSG extracts to protect against oxidant-induced GSH depletion.

The black phenolic extract BW2, BD1 and BD2 (0.5% v/v) and pale phenolic extracts PD1-3, PW2 and PW3 (4% v/v) protected against an oxidant-induced decrease in SOD activity (Figure 2B). Extracts BW1 and PW1, which were not treated with enzyme, did not protect against the H_2O_2 induced depletion in SOD activity. This would indicate that the extraction conditions used may have enhanced the ability of extracts to augment the antioxidant enzyme activity of the cells. The antioxidant effects of cereal extracts have previously been investigated by Qingming *et al*. (2010) and Giriwono *et al*. (2010). Qingming *et al*. (2010) found that the administration of a malt extract from barley to D-galactose-treated mice increased the activities of SOD and glutathione peroxidase in brain and liver tissue. Giriwono *et al*. (2010) found that an extract of fermented barley improved the expression and enzyme activity of glutathione peroxidase 1 and CAT in the livers of chronic ethanol-fed female Wistar rats but there was no significant effect on hepatic SOD activity. The activity of catalase in HepG2 cells was reduced to approximately 40% of the control following exposure to H_2O_2 (Figure 2C). Similar to the results of the SOD activity, neither BW1 nor PW1 (prepared in the absence of enzymes) protected against the H_2O_2-induced reduction in catalase activity. This again suggests that carbohydrase enzymes may be required to release active phenolic compounds found in BSG. Extracts BW2, BW4 and PW3 significantly ($P<0.05$) protected against the oxidant induced decrease in catalase activity.

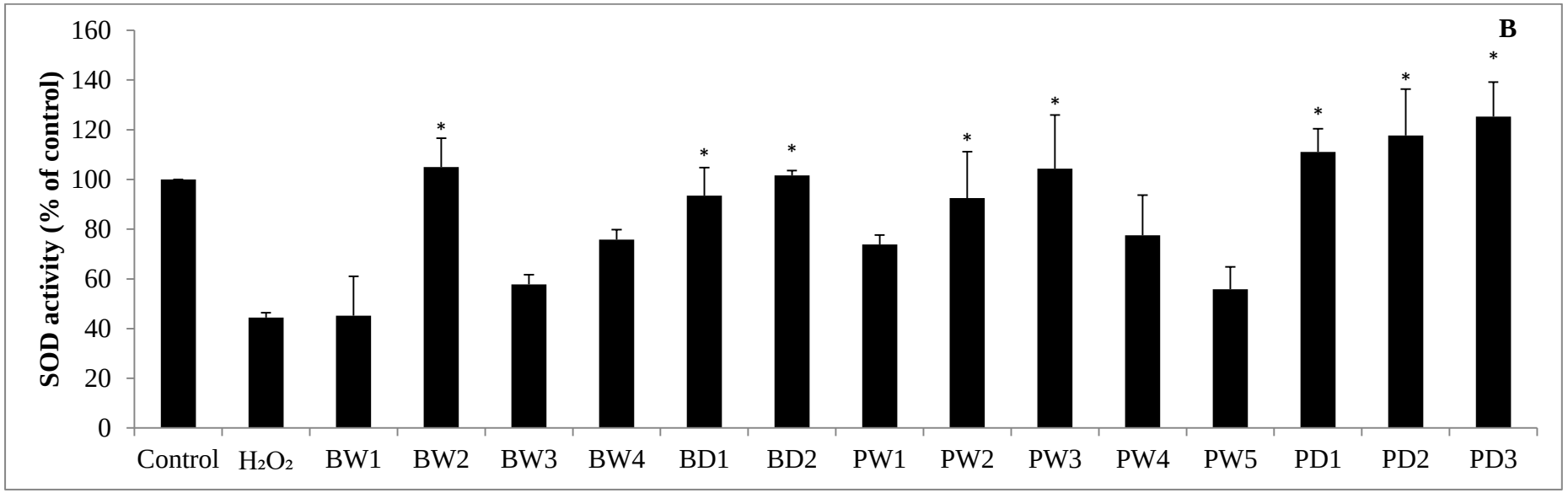


Figure 2B: Antioxidant potential of BSG black [0.5% (v/v)] and pale [4% (v/v)] phenolic extracts as assessed by their ability to protect superoxide dismutase (SOD) activity against H_2O_2-induced oxidative stress in HepG2 cells. BW1-4 represent extracts from black, wet BSG; BD1-2 represents extracts from black, dry BSG; PW1-5 represents extracts from pale, wet BSG and PD1-3 represents extracts from pale, dry BSG. Values represent the mean ± SE of at least three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * Statistically significant difference between sample and H_2O_2 control ($P<0.05$).

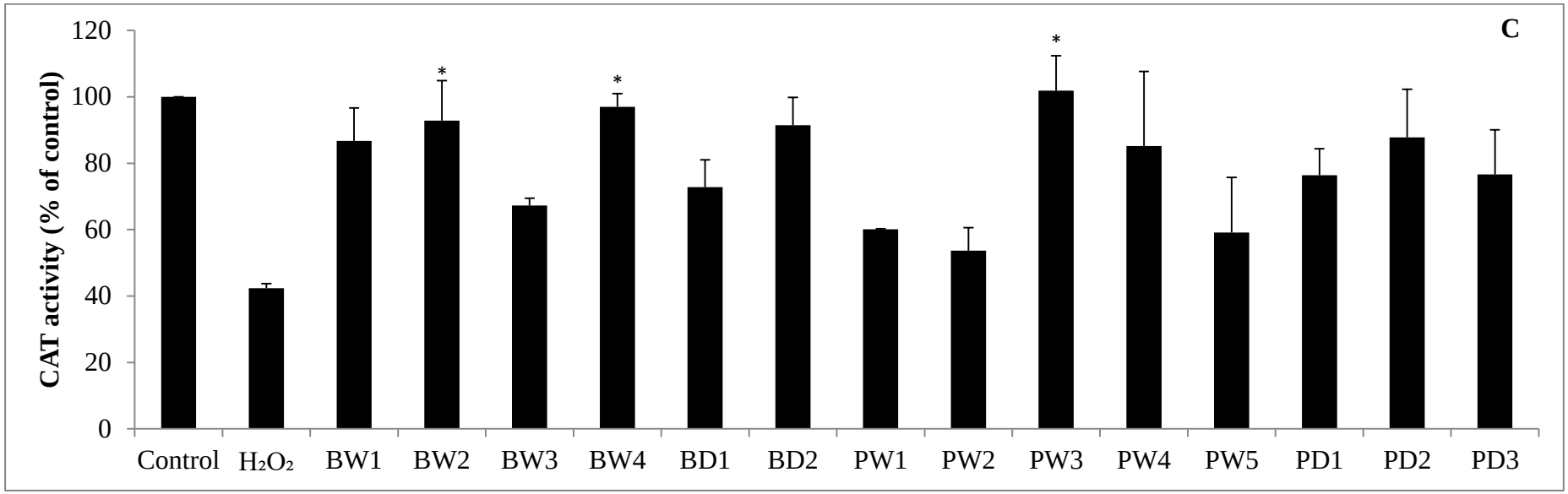


Figure 2C: Antioxidant potential of BSG black [0.5% (v/v)] and pale [4% (v/v)] phenolic extracts as assessed by their ability to protect catalase (CAT) activity against H_2O_2-induced oxidative stress in HepG2 cells. BW1-4 represent extracts from black, wet BSG; BD1-2 represents extracts from black, dry BSG; PW1-5 represents extracts from pale, wet BSG and PD1-3 represents extracts from pale, dry BSG. Values represent the mean ± SE of at least three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * Statistically significant difference between sample and H_2O_2 control ($P<0.05$).

Conclusions

The DNA protective and cellular antioxidant potential of a series of novel BSG extracts, prepared using carbohydrases, were investigated. BSG extracts protected against DNA damage however, the use of enzymes in the extraction procedure did not enhance the DNA protective effects of the extracts. In general, BSG extracts prepared in the presence of enzymes were more effective in protecting against a depletion of cellular antioxidant GSH content and SOD and CAT activity in oxidant-challenged HepG2 cells than the extracts prepared in the absence of the enzymes. Further research is required in order to fully characterise the bioactive constituents of the extracts however, the findings of this study indicate that enzyme-extraction could be used in the production of novel, natural, plant-derived bioactive components for use in the food industry as an alternative to conventional solvent extraction.

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Chapter 4

In vitro antioxidant potential of water drinks fortified with brewers' spent grain phenolic extract before and after *in vitro* digestion.

Abstract

Brewers' spent grain (BSG) is the residual solid fraction left after the brewing process. Owing to its phenolic content, extracts from BSG have demonstrated antioxidant effects in a number of different *in vitro* experimental models. Here, the ability of enzyme-extracted BSG phenolic extracts to enhance the antioxidant potential of flavoured water drinks before and after *in vitro* digestion was investigated. The antioxidant activities of BSG phenolic extracts was measured by total phenolic content (TPC), 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay and Ferric reducing antioxidant power (FRAP). The antioxidant potential of water drinks fortified with BSG phenolic extracts was assessed by DPPH, FRAP and Oxygen Radical Absorbance Capacity (ORAC). Results demonstrated that addition of phenolic extracts did not significantly increase antioxidant activity of commercially available antioxidant-containing flavoured water drinks.

Introduction

With a heightened understanding of the benefits of antioxidant compounds, there is a greater demand from consumers to increase the development of beverages rich in antioxidants (Loizzo *et al*., 2012). Water is the most consumed beverage in the world. Flavoured water has become a prominent product on the marketplace. It typically contains flavours, juices, bioactive compounds, preservatives and/or sweeteners that give singular tastes and smells accepted by consumers (Fatima Barroso *et al*., 2009). These drinks are supplemented with antioxidant substances capable of providing protection against oxidative stress (Loizzo *et al*., 2012).

The most common by-products of the brewing industry are spent grain, spent hops and yeast (Mussatto *et al*., 2006). Brewers' spent grain (BSG) includes the seed coat–pericarp–husk fractions of the original barley grain. As a by-product, BSG represents a useful source of compounds that have potential uses as functional, bioactive ingredients in human nutrition (Lynch *et al*., 2016). BSG has been shown to be a source of valuable phenolic compounds, hydroxycinnamic acids (McCarthy *et al*., 2013a), which have demonstrated antioxidant activity in relation to several diseases where oxidative stress is a prominent feature,

including inflammatory injury, cancer, and cardiovascular diseases (Teixeira *et al*., 2013). Phenolic-enriched extracts of BSG have previously demonstrated cellular antioxidant activities (McCarthy *et al*., 2012; McCarthy *et al*., 2014; Crowley *et al*., 2017).

It is important that phenolic extracts from BSG demonstrate an ability to retain their bioactivity during digestion (McCarthy *et al*., 2013b). *In vitro* digestion models can be used to quickly evaluate the stability of food ingredients during digestion, as an alternative to animal and human trials (Hur *et al*., 2011). This provides a truer reflection of the health benefits of bioactive compounds, revealing the amount of a compound available for absorption. Bioaccessibility entails the amount of compound that is released from a food matrix into the gut. Phenolic compounds released via enzymatic digestion in the gastrointestinal tract are potentially available for absorption (Chandrasekara and Shahidi, 2012).

The aim of this particular research was to assess the antioxidant potential of flavoured waters fortified with select phenolic extracts of BSG and secondly, to assess the stability of the BSG extracts during simulated gastrointestinal digestion. The selected BSG phenolic extracts had previously demonstrated cellular antioxidant potential, reducing hydrogen peroxide (H_2O_2)-induced DNA damage in U937 cells and protecting against H_2O_2-induced decreases in glutathione content, superoxide dismutase and catalase activity (Crowley *et al*., 2017).

Materials and Methods

Materials

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich Ireland Limited, Co. Wicklow, Ireland.

Preparation of phenolic extracts

Pale BSG was the residual solid fraction retained following the removal of wort during beer production in the brewing process. BSG was stored in 1 kg quantities

in polyethylene bags which were vacuum packed and maintained at −20°C. To generate phenolic extracts, BSG was sheared in distilled H_2O (1:10) at 24,000 rpm for 2 minutes. The supernatant was then incubated with carbohydrases (Shearzyme, Ultraflo Max and/or Depol 740L) for either 240 or 540 minutes, at 50°C and pH5 or pH7 (Table 1). Phenolic preparations from pale BSG were designated PD1, PD2, PD3 and PW3.

Total phenolic content (TPC)

The Folin–Ciocalteu assay as described previously (Singleton and Ross, 1965) was used to measure the antioxidant capacity of BSG phenolic extracts. This assay is based on the capacity of a compound to reduce the Folin–Ciocalteau reagent (FCR), generating a yellow to blue/green colour change that is measured spectrophotometrically at 765 nm. The TPC method is non-specific to phenols and measures any reducing agent. Results were expressed as milligram gallic acid equivalents per ml phenolic extract (mg GAE mL^{-1}).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The DPPH assay was performed according to the method illustrated by Brand-Williams *et al*. (1995). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) concentrations from 0.04 to 0.40 μM were used to prepare a standard curve for calibration. To allow for coloured samples, colour blanks were used consisting of 100 μl sample and 3.9 ml methanol (MetOH). Absorbance at 515 nm of all samples was measured following 30 min incubation with the DPPH reagent (0.0238 μg mL^{-1}). The assay measures antioxidant potential based on a compound's ability to scavenge the DPPH radical by hydrogen donation. Results were expressed as %DPPH inhibition.

Table 1: Extraction procedure used for the generation of BSG phenolic extracts

Sample code	BSG type	Enzyme	Enzyme volume (µL g^{-1} BSG_{dw})	Temperature (°C)	pH	Extraction time (minutes)
PD1	Pale Dry	Depol 740L	10	50	5	240
PD2	Pale Dry	Depol 740L	100	50	7	240
PD3	Pale Dry	Shearzyme, Ultraflo Max & Depol 740L	100 (each)	50	5	540
PW3	Pale Wet	Depol 740L	100	50	7	240

Pale BSG was the residual solid fraction retained following the removal of wort during beer production in the brewing process. Wet BSG contains approximately 70-80% water. Dry BSG contains <10% moisture.

Ferric reducing antioxidant power (FRAP)

The FRAP assay (Benzie and Strain, 1996), was used to determine the reducing power of the samples. The assay is based on the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}), which can be measured spectrophotometrically at 593 nm. To account for sample colour, colour blanks were prepared with 100 µl sample and 2.9 ml distilled H_2O where appropriate. For calibration, 0–100 µM ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) was used and results were expressed as µM FRAP.

Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay was performed as described by Kittiphattanabawon *et al*. (2012) with some modifications. The samples were diluted 1:10 in 75 mM phosphate buffer (pH 7.0). The prepared sample (25 µL) was loaded onto white polystyrene, nontreated 96-well microplate (Costar Corning Inc., Corning, NY, USA). Only the internal wells of the microplate were used. Then 50 µL of 0.04 µM fluorescein dissolved in 75 mM phosphate buffer (pH 7.0) was added to the sample. The loaded microplate was allowed to equilibrate at 37°C for 20 min in a microplate reader (Thermo Scientific Varioskan® Flash Multimode Reader, Fisher Scientific UK Ltd., Leicestershire, UK). The reaction was started by the addition of 100 µL of 221 mM AAPH. The reaction was performed at 37°C. The fluorescence intensity was measured every 5 min for 25 cycles with excitation and emission filters of 485 and 535 nm, respectively. The control was prepared in the same manner, except that 75 mM phosphate buffer (pH 7.0) was used instead of the sample. The kinetic curve (AUC) of the samples was plotted between relative fluorescence intensity and time. Trolox (0–100 µM) was used as the standard. The ORAC was expressed as µmol Trolox equivalents (TE).

Preparation of fortified drinks

Five commercially available water drinks were purchased from a local supermarket; Ballygowan Still Natural Mineral Water (Britvic Soft Drinks Ltd.,

UK), Tesco Lemon And Lime Still Flavoured Water (Tesco, UK), Volvic Touch Of Fruit Lemon and Lime (Danone Waters (UK and Ireland) Ltd., UK), Sobe V Water Pomegranate And Blueberry (Britvic Soft Drinks Ltd., UK) and Glaceau Vitaminwater XXX Triple Berry Acai-Blueberry-Pomegranate (Coca-Cola Enterprises Ltd., UK) (Table 2). Owing to the presence of known antioxidant compounds in both products, the Sobe and Glaceau water drinks were used as positive controls for the purpose of this research. Samples were aliquoted into 15 ml centrifuge tubes and stored at −20°C until analysis. For fortified samples, BSG phenolic extracts, PD1-3 and PW3, were added at 10% (v/v) to a final volume of 5 ml prior to freezing. The selection of these extracts was based on their pale colour (therefore being unable to influence the colour of the water drinks) and their antioxidant potential demonstrated in cell culture models (Crowley *et al*., 2017). Previous research has been carried out using BSG phenolic extracts at concentration of 10% v/v (McCarthy *et al*., 2013b). The antioxidant activities of all samples were analysed before and after *in vitro* digestion.

In vitro digestion

A SGID model, as previously described (McCarthy *et al*., 2015) was utilised to simulate human digestion. Briefly, for the gastric digestion 2 mL sample and 1 mL Pepsin (0.04 g mL^{-1} in 0.1M HCl) were added to 12 mL Hank's balanced salt solution (HBSS) and the pH was adjusted to 2 using 0.1M HCl, to simulate the physiological conditions of the stomach. Samples were incubated for 1 h in a shaking waterbath (Grant OLS 200, Grant Instruments, Cambridge, UK) at 95 rpm and 37^{o}C. To simulate duodenal digestion, 100 µL Pancreatin (0.08 g mL^{-1} in HBSS) and 200 µL bile salts (0.2 g glycodeoxycholate, 0.125 g taurodeoxycholate and 0.2 g taurocholate in 5 mL HBSS) were added, the pH was adjusted to 7.4 using 1N NaOH and samples were incubated in a shaking waterbath (95 rpm) at 37^{o}C for 2 h. Samples were centrifuged at 5,525 x g for 1 h and the supernatant (digestate) was isolated, aliquoted, blown with N_2 gas and stored at -20^{o}C.

Table 2: Water drinks ingredients list

Water drink	Ingredients list (as per label)
Ballygowan Still Natural Mineral Water	Natural mineral water
Tesco Lemon And Lime Still Flavoured Water	Mineral Water, Acid (Citric Acid), Flavourings, Preservatives (Potassium Sorbate, Dimethyl Dicarbonate), Sweetener (Sucralose)
Volvic Touch Of Fruit Lemon And Lime	Volvic Natural Mineral Water (92%), Sugar (5.4%), Acid (Citric Acid), Natural Lemon and Lime Flavouring, Preservative (Potassium Benzoate)
Sobe V Water Pomegranate And Blueberry	Spring Water, Natural Blueberry Flavouring with Other Natural Flavourings, Acid (Citric Acid), Vitamins: C, E, Carrot and Hibiscus Concentrates, Sweetener (Steviol Glycosides), Minerals: Zinc Sulphate, Sodium Selenite, Burdock Root Extract
Glaceau Vitaminwater XXX Triple Berry Acai-Blueberry-Pomegranate	Spring Water, Fructose, Sugar, Citric Acid, Vitamins (C, Niacin, E, Pantothenic Acid, B6), Fruit and Vegetable Concentrates (Blueberry, Carrot), Fruit Extracts (Apple, Blueberry, Pomegranate, Acai), Natural Flavourings, Sweetener (Steviol Glycosides), Mineral Salt (Zinc Gluconate)

Analysis of the antioxidant potential of flavoured waters

The antioxidant activities of fortified flavour water were determined before and after digestion by DPPH, FRAP and ORAC.

Statistical Analysis

All data represent the mean ± standard error (SE) of at least three independent experiments. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test as analysed using GraphPad Prism 4 (GraphPad software, La Jolla, CA).

Results

TPC, DPPH and FRAP measurements of BSG phenolic extracts

Table 3 shows the antioxidant potential of BSG phenolic extracts as measured by TPC, DPPH and FRAP. There were no statistically significant differences between the extracts. The BSG phenolic extract PD3 demonstrated the highest phenolic acid content as measured by the TPC assay (0.03 mg GAE mL^{-1}), whereas extract PW3 had the lowest TPC measurement (0.01 mg GAE mL^{-1}) (Table 3). Additionally, extract PW3 elicited the lowest antioxidant effect when assessed by the DPPH assay. The highest DPPH inhibition was identified with extract PD1 (25.80%). Similar to the TPC measurement, BSG extract PD3 produced the highest FRAP value (115.66 µM). Correlation statistics suggest positive correlations between the TPC and FRAP assays (R^2=0.62). The correlation between the TPC and DPPH assays (R^2=0.30) and DPPH and FRAP assays was considerably lower (R^2=0.01).

Antioxidant activity of fortified drinks before in vitro digestion

The ability of phenolic extracts to enhance the antioxidant potential of flavoured water drinks was investigated. % DPPH inhibition of between 15.76-25.98% was

Table 3: Total phenolic content (TPC), DPPH radical inhibition and Ferric reducing antioxidant power (FRAP) of brewers' spent grain (BSG) phenolic extracts.

Sample	TPC (mg GAE mL^{-1})	DPPH inhibition (%)	FRAP (µM)
PD1	0.02 ± 0.01	25.80 ± 4.15	114.15 ± 5.65
PD2	0.02 ± 0.01	18.71 ± 2.76	114.91 ± 6.60
PD3	0.03 ± 0.01	20.24 ± 5.76	115.66 ± 6.40
PW3	0.01 ± 0.01	12.89 ± 1.93	114.39 ± 5.77

Data represents mean ± SE of at least three independent experiments.

achieved by unfortified control waters (Table 4). Addition of BSG phenolic extracts increased the % DPPH inhibitory activity of all the water drinks assessed. However, none of these increases were statistically significant. Statistically significant increases in % DPPH inhibition were noted with the positive controls, Sobe V Water and Glaceau Vitaminwater. The water drinks were used as positive controls owing to their added antioxidant content (Table 2). Addition of the BSG phenolic extracts led to no change in the FRAP activity of the water drinks.

Similar to the strong DPPH inhibitory activity, Sobe V Water and Glaceau Vitaminwater demonstrated significantly higher FRAP activity compared to control waters. Phenolic extract fortification of both Ballygowan and Volvic waters led to non-significant increases in ORAC values. A non-significant decrease in ORAC values was noted with the addition of phenolic extracts to Tesco water.

Antioxidant activity of fortified drinks after in vitro digestion

The impact of simulated gastrointestinal *in vitro* digestion (SGID) on the antioxidant activity of BSG phenolic extract-fortified water drinks can be seen in Table 5. Non-significant decreases in % DPPH inhibitory activity was noted with all phenolic extract fortified-water drinks assessed. The antioxidant containing-water drinks Sobe V Water and Glaceau Vitaminwater showed statistically significantly lower % DPPH inhibitory activity following SGID. There was quite a lot of variation in the FRAP values of water drinks subjected to SGID. For example, SGID led to non-significant increases in the FRAP values of Volvic waters supplemented with BSG phenolic extracts PD1-PD3 and PW3, whereas Tesco waters supplemented with extracts PD1, PD2 and PW3 demonstrated non-significantly lower FRAP values. Significant decreases in the FRAP values of Sobe V Water and Glaceau Vitaminwater were noted after SGID. Of all the water drinks assessed, no significant changes in ORAC values, as a result of SGID, were noted.

Table 4: Antioxidant activity of flavoured mineral waters supplemented with BSG phenolic extracts.

Water sample		DPPH inhibition (%)	FRAP (μM)	ORAC (μM TE)
Ballygowan	Control	18.35 ± 9.31	84.18 ± 2.62	218.59 ± 151.28
	10% (v/v) PD1	24.66 ± 5.93	79.69 ± 2.69	451.67 ± 57.23
	10% (v/v) PD2	30.24 ± 7.73	80.31 ± 1.59	563.37 ± 94.63
	10% (v/v) PD3	25.33 ± 5.35	83.36 ± 2.53	530.77 ± 67.67
	10% (v/v) PW3	24.28 ± 8.59	81.18 ± 2.45	532.95 ± 89.77
Tesco	Control	15.76 ± 4.36	84.51 ± 2.11	489.14 ± 70.78
	10% (v/v) PD1	31.89 ± 4.41	77.77 ± 2.04	321.64 ± 85.55
	10% (v/v) PD2	33.27 ± 5.69	84.09 ± 1.86	418.25 ± 124.66
	10% (v/v) PD3	34.69 ± 5.29	79.18 ± 2.08	280.38 ± 109.95
	10% (v/v) PW3	29.68 ± 5.37	81.70 ± 2.34	487.56 ± 66.14
Volvic	Control	25.98 ± 5.91	82.03 ± 2.66	345.47 ± 121.34
	10% (v/v) PD1	30.23 ± 3.93	78.13 ± 1.39	543.11 ± 91.90
	10% (v/v) PD2	32.33 ± 6.61	80.49 ± 3.40	449.75 ± 117.43
	10% (v/v) PD3	33.84 ± 6.25	80.54 ± 2.60	465.16 ± 95.55
	10% (v/v) PW3	28.15 ± 6.24	81.72 ± 1.72	521.90 ± 117.57
	Glaceau	87.77 ± 1.19*	107.58 ± 4.06*	499.93 ± 40.92

Sobe	90.01 ± 3.46*	102.23 ± 1.93*	442.89 ± 40.46

Data represents mean ± SE of at least three independent experiments. Statistical analysis by ANOVA followed by Tukey's multiple comparison test. * Denotes statistically significant difference ($P<0.05$) within a column between control water and supplemented/unsupplemented water.

Table 5: Antioxidant activity of flavoured mineral waters supplemented with BSG phenolic extracts, following *in vitro* digestion.

Water sample		DPPH inhibition (%)	FRAP (μM)	ORAC (μM TE)
Ballygowan	Control	3.73 ± 2.37	79.66 ± 1.97	407.51 ± 78.63
	10% (v/v) PD1	9.10 ± 5.40	79.93 ± 3.43	521.15 ± 46.38
	10% (v/v) PD2	9.43 ± 6.99	77.93 ± 1.54	589.18 ± 54.65
	10% (v/v) PD3	10.51 ± 5.58	78.40 ± 0.59	538.73 ± 33.52
	10% (v/v) PW3	9.62 ± 6.28	78.69 ± 0.94	496.67 ± 30.42
Tesco	Control	3.62 ± 2.19	76.78 ± 1.15	459.42 ± 51.90
	10% (v/v) PD1	11.52 ± 6.10	77.59 ± 0.92	374.65 ± 44.44
	10% (v/v) PD2	9.84 ± 6.03	77.96 ± 1.42	507.73 ± 97.32
	10% (v/v) PD3	10.79 ± 5.80	82.21 ± 2.13	365.74 ± 78.03
	10% (v/v) PW3	6.16 ± 4.73	79.39 ± 1.47	533.15 ± 99.85
Volvic	Control	2.61 ± 1.35	78.40 ± 1.07	352.97 ± 31.55
	10% (v/v) PD1	11.30 ± 6.60	86.63 ± 7.24	515.66 ± 75.39
	10% (v/v) PD2	9.76 ± 5.61	82.88 ± 3.15	552.59 ± 96.71
	10% (v/v) PD3	8.16 ± 5.10	84.90 ± 5.07	484.14 ± 57.24
	10% (v/v) PW3	11.21 ± 6.27	86.42 ± 8.23	548.18 ± 91.53
	Glaceau	23.63 ± 16.23[a]	79.63 ± 1.47[a]	495.99 ± 26.70

Sobe	8.31 ± 6.78[a]	79.09 ± 1.05[a]	518.54 ± 16.15

Data represents mean ± SE of at least three independent experiments. Statistical analysis by ANOVA followed by Tukey's multiple comparison test. [a] Denotes statistically significant difference ($P<0.05$) within a column between undigested (Table 4) and digested values.

Discussion

A functional beverage is described as a drink product that is ready to drink and which contains non-traditional ingredients. Popular ingredients include herbs, vitamins, minerals, amino acids or additional raw fruit or vegetable ingredients. Such drink products can provide specific health benefits that go beyond general nutrition (Loizzo *et al*., 2012).

The potential of polyphenols to protect against chronic disease development has encouraged food companies to create drinks containing antioxidants that appeal to consumers. Such drinks include vitamin enhanced waters (VEWs), as well as fruit and berry juices. A large number of VEWs exist on the market with some products claiming to contain high antioxidant levels and therefore high antioxidant capacity (Donnelly *et al*., 2010). BSG is a co-product of the brewing industry which has been added as an ingredient to certain foods (Ktenioudaki *et al*., 2012; Guo *et al*., 2014). It has also demonstrated promising effects as a potential functional ingredient of foods such as cranberry juice, yoghurt, snack bars and milk (McCarthy *et al*., 2013a; McCarthy *et al*., 2015; Crowley *et al*., 2015). Recently, BSG phenolic compounds generated using a carbohydrase-extraction method, demonstrated antioxidant potential in cell culture models (Crowley *et al*., 2017). The aim of this research was to assess the ability of select enzyme-extracted BSG phenolic extracts to enhance the antioxidant activity of water drinks.

The initial assessment of the antioxidant potential of the BSG phenolic extracts involved the use of the *in vitro* antioxidant assays; TPC, DPPH and FRAP. The extracts were derived from pale BSG which is known as a source of hydroxycinnamic acids (McCarthy *et al*., 2013a). Here extract PD3 demonstrated the highest TPC and FRAP measurements, while extract PD1 was the most effective extract at inhibiting DPPH. The TPC and FRAP assays showed good correlation (R^2=0.62), suggesting that the ability to reduce ferric ions to the ferrous form is related to the antioxidant content of the extracts. Similar positive correlations between the methods have been also highlighted in other studies (Hashim *et al*., 2013; Ku *et al*., 2014). The ability of antioxidants to reduce ferric tripyridyltriazine to ferrous tripyridyltriazine can be measured using the FRAP

assay (Loizzo *et al*., 2012). The TPC method is of course, not specific to phenolic compounds as it measures any compound which is capable of reducing Folin – Ciocalteu reagent (Huang *et al*., 2005). Previous research on the *in vitro* antioxidant potential of alkaline-extracted BSG phenolic extracts demonstrated higher average TPC measurements compared with extracts analysed here (McCarthy *et al*., 2012; McCarthy *et al*., 2013a). In contrast to this, the DPPH inhibitory activity of enzyme-extracted BSG phenolic extracts was on average higher than that of alkaline-extracted BSG phenolic extracts. This may highlight the effectiveness of carbohydrase enzymes at releasing antioxidant components in the BSG. DPPH is a radical which has a UV-vis absorption maximum of 515 nm and can be reduced, leading to a colour change which can be recorded by spectrophotometry. Many antioxidants may be non-reactive or may react slowly with DPPH, yet react quickly with peroxyl radicals (Huang *et al*., 2005).

The ability of phenolic extracts to enhance the antioxidant potential of flavoured water drinks was investigated. Similar research in the past has assessed the ability of both BSG phenolic and protein extracts to increase the antioxidant and anti-inflammatory potential of different food formulations (McCarthy *et al*., 2013a; McCarthy *et al*., 2015; Crowley *et al*., 2015). Results presented here demonstrated that addition of phenolic extracts to water drinks did not significantly increase antioxidant activity. Increases in both DPPH scavenging and FRAP activity were noted with Glaceau and Sobe drinks. Glaceau Vitaminwater contains among other vitamins and minerals, vitamin C and E as well as zinc which are all known as antioxidants (Padayatty *et al*., 2003; Traber and Atkinson, 2007; and Prasad, 2014). Similarly, Sobe V Water contains these nutrients as well as selenium which is also known to play a role as an antioxidant (Tinggi, 2008). Both drinks contain concentrates of carrot, which is a rich source of antioxidant carotenoids such as β-carotene (Sharma *et al*., 2012). Sobe V Water contains a concentrate of Hibiscus, the leaves of which are consumed in many countries and have been found to contain high levels of polyphenols, such as chlorogenic acid, quercetin and kaempferol glycosides (Zhen *et al*., 2016). The drink also contains a root extract from the perennial herb, burdock, which has demonstrated *in vitro* antioxidant activity and contains tannin, caffeic acid and chlorogenic acid (Predes *et al*., 2011; Chan *et al*., 2011). The Glaceau Vitaminwater also contains extracts

from apple, pomegranate, blueberry and acai. Zhang *et al*. (2016) found that the flavonoids phloridzin and phloretin were the principal components responsible for the antioxidant properties of an apple extract. Pomegranate extracts have also been shown to have very high antioxidant activity (Salgado *et al*., 2012; Kalaycioglu and Erim, 2017). Both blueberries and acai are rich in anthocyanins (Wang *et al*., 2017; Santos *et al*., 2014). The ORAC and PSC assays were used to show the high antioxidant activity of blueberry extracts *in vitro* and in cellular systems (Wang *et al*., 2017). The açaí fruit is derived from *Euterpe oleracea* and Santos *et al*. (2014) showed that an açaí extract protected astrocytes against manganese neurotoxicity. The antioxidant capability of Vitaminwater brand beverages has previously been reported by Donnelly *et al*. (2010) and Hong *et al*., (2016). Also of note here are the antioxidant effects of the unfortified (control) water drinks. These drinks contain mineral water and, in the case of the Tesco and Volvic drinks, flavourings and preservatives. A possible reason for the antioxidant effect is that the mineral content of these waters is contributing to the antioxidant effect. Nappi *et al*. (2011) demonstrated the positive antioxidant role of hydric supplementation with a "Lete"mineral water in human subjects. A possible reason for the lack of an enhancement effect with fortification of the drinks may be connected to the low concentration (10% v/v) added to the water drinks. The antioxidant effects of the phenolic extracts are likely to have been diluted. The 10% (v/v) concentration was selected based on previous research (McCarthy *et al*., 2013a), where addition of phenolic extracts to cranberry juice increased the FRAP activity of the drink.

As can be seen in Table 5, digestion did not significantly impact on the antioxidant activity of the fortified drinks. However significant decreases in the DPPH scavenging and FRAP activity of both Glaceau and Sobe after digestion were noted. Other studies which have employed SGID to assess the bioaccessibility of phenolic compounds found similar results. Schulz *et al*. (2017) assessed the minerals and phenolic contents, as well as antioxidant capacity of juçara fruit (*Euterpe edulis* Martius) before and after the *in vitro* gastrointestinal digestion and noted a decrease in DPPH scavenging capacity and FRAP after SGID (Schulz *et al*., 2017). Similarly, Celep *et al*. (2015) noted decreases in the H_2O_2-scavenging activities and total antioxidant capacities of wine samples after

in vitro gastrointestinal digestion. The authors speculated that lower concentrations of phenolic compounds compared to undigested samples and pH variations during digestion were responsible for the lower antioxidant activity of digested samples. In contrast to this, Chandrasekara and Shahidi (2012) found that the TPC, total flavonoid content and trolox equivalent antioxidant capacity values of millet grains increased following *in vitro* digestion. It was suggested that a likely reason for this was the transformation of compounds present in the raw grain during the digestive process. Previous research on BSG extract-fortified foods has demonstrated positive results with regards to their ability to retain bioactivity following *in vitro* digestion. McCarthy *et al*. (2015) demonstrated that digestates of foods supplemented with BSG phenolic extracts significantly reduced oxidant-induced DNA damage in Caco-2 cells, as well as attenuating IL-4, IL-10 and IFN-γ production in stimulated Jurkat T cells. Similarly, milk fortified with BSG protein hydrolysates decreased IL-6 production in stimulated Jurkat T cells, following *in vitro* digestion (Crowley *et al*., 2015).

Conclusions

In conclusion, the addition of BSG phenolic extract does not significantly enhance the antioxidant potential of water drinks, either prior to or after *in vitro* digestion.

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Chapter 5

Effects of varying the carbohydrase enzymes on the production of anti-inflammatory and antioxidant hydrolysates from brewers' spent grain (BSG).

Abstract

Brewers' spent grain (BSG) is the residual fraction left over from the brewing process and is an excellent source of bioactive compounds. Enzyme extraction methods may be favoured over solvent extraction procedures due to their specificity and mild reaction conditions. Here the potential of using carbohydrase (Shearzyme, Ultraflo Max, and Depol 740 L) and proteinase (Alcalase, Brewers Clarex and Flavourzyme) enzymes to extract BSG protein hydrolysates with anti-inflammatory and antioxidant activities was investigated. The impact of BSG protein hydrolysates on cytokine production in both concanavalin A (ConA)-treated Jurkat T cells and lipopolysaccharide (LPS)-stimulated RAW 264.7 cells, DNA damage in H_2O_2-treated U937 cells and GSH content, SOD and CAT activity of H_2O_2-treated HepG2 cells was assessed. The most effective enzyme treatment yielding bioactive BSG hydrolysates was seen with the carbohydrase combination of Shearzyme and Ultraflo Max and proteinase combination of Alcalase, Brewers Clarex and Flavourzyme. The unhydrolysed fraction also demonstrated both anti-inflammatory and antioxidant activity, suggesting that other components present in the BSG may be responsible for its bioactivity.

Introduction

It is now well established that food or food waste materials may contain components that have biological activities above the basic nutritional value. This has led to increased interest from the food industry, particularly concerning the development of so-called nutraceuticals (Cheli and Baldi, 2011). In this regard, food or food waste-derived biologically active peptides (bioactive peptides) offer potential as functional food components that can be used to treat a variety of medical conditions including inflammation, hypertension, diabetes and cardiovascular disease. The inclusion of bioactive peptides through food is an appealing alternative to delivering synthetic drugs. In developing countries, these naturally-occurring bioactive peptides are often more cost-effective and more widely available than synthetic drugs (Maestri *et al*., 2016). Hydrolysates have been described as mixtures consisting of peptides and amino acids which are generated by enzyme, acid or alkali treatment of proteins or by fermentation of

proteins (Sarmadi and Ismail, 2010). Bioactive peptides differ, in that they are several linked amino acids purified from hydrolysates (Sarmadi and Ismail, 2010). Hydrolysates are generally easier to obtain than purified peptides (Maestri *et al*., 2016).

The immune system serves as the first line of defence against pathogens and aids in protecting the body against potential infections. Proper immune functioning is important for the prevention and elimination of infections (Santiago-Lopez *et al*., 2016). Peptides obtained from soybeans and milk have demonstrated anti-inflammatory effects on intestinal inflammation, with the inhibition of pro-inflammatory cytokines and chemokine expression (Cicero *et al*., 2017). Oxidative stress is an imbalance between oxidants and antioxidants (Cheli and Baldi, 2011). Reactive oxygen species (ROS), produced from the mitochondrial respiratory chain, are the predominant contributors to oxidative damage. Antioxidants are molecules with an ability to stabilise or deactivate the harmful effects of free radicals, such as ROS (Cheli and Baldi, 2011). Antioxidative peptides are capable of reducing the damage caused by ROS, resulting in the decrease of essential fatty acid peroxidation (Maestri *et al*., 2016).

Brewers' spent grain (BSG) is the insoluble part of the barley grain, constituting the seed coat–pericarp–husk layers that encased the original cereal grain, which is left over from the brewing process (Lynch *et al*., 2016). BSG is the residual fraction following the mashing step and because of the large amount produced annually and current commercial value, there is a lot of interest in exploiting this nutritionally valuable co-product (Lynch *et al*., 2016). BSG has proven to be an excellent source of bioactive compounds obtained by different extraction methods, such as solid–liquid extraction, microwave-assisted extraction, alkaline extraction and enzymatic extraction (Mussatto, 2014). With the presence of different substituted groups and various bond linkage types of the BSG polymers, such as the hemicellulose fraction, enzymes with diverse activities are required for complete polymer hydrolysis (Lynch *et al*., 2016). Enzyme extraction methods may be favoured over solvent extraction procedures due to their specificity and mild reaction conditions (Forssell *et al*., 2008). BSG (15–25% w/w protein) is currently being researched as a source of protein concentrate or hydrolysates with techno-functional applications and/or health benefits (Lynch

et al., 2016). Recent studies on alkaline-extracted protein hydrolysates generated from BSG suggest that it is a source of anti-inflammatory and antioxidant peptides (McCarthy *et al*., 2013a; McCarthy *et al*., 2013b). The purpose of this research was to investigate the potential of using carbohydrase (Shearzyme, Ultraflo Max and Depol 740L) and proteinase (Alcalase, Brewers Clarex and Flavourzyme) enzymes to extract BSG protein hydrolysates with anti-inflammatory and antioxidant activities.

Materials and Methods

Materials

BSG was obtained from a single batch which was brewed in November 2009, vacuum-packed and stored at -20^{o}C in polypropylene bags until use. Shearzyme 500 L (declared activity 500 Fxu g21) and Ultraflo Max (declared activity 470 Fxu g21) were sourced from Novozymes A/S, Bagsværd, Denmark. Depol 740L (declared activity 945 u g21) was purchased from Biocatalysts Ltd., Cardiff, UK. Alcalase 2.4L and Flavourzyme® 500L were purchased from Sigma Chemical Co. (Dorset, UK). Brewer's Clarex™ (*Aspergillus niger* prolyl endoproteinase) was received from Dutch State Mines (DSM, Heerlen, Netherlands). Jurkat T cells, RAW 264.7 murine macrophages, U937 cells and HepG2 cells were obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). All other chemicals were purchased from Sigma, unless otherwise stated.

Generation of the enzyme-extracted hydrolysates

The enzyme-extracted hydrolysates were prepared by shearing BSG in distilled H_2O (1:10) at 24000 rpm for 2 minutes using an Ultra Turrax®T25 high-performance disperser (IKA® Werke GmbH & Co. KG, Staufen, Germany). The BSG suspension was then incubated with carbohydrases: Shearzyme 500 L, Ultraflo Max and/or Depol 740L for either 240 or 540 minutes, at 50°C (Table 1). Samples were centrifuged at 2700 g for 20 minutes at 10°C (Hettich Zentrifugen

Table 1: Summary of methods used to prepare brewers' spent grain (BSG) protein hydrolysates A-J.

Sample	Description	Carbohydrase enzyme	Protease enzymes	pH
A	Unhydrolysed BSG protein	No enzyme	No enzyme	_
B	BSG protein hydrolysate	Shearzyme, Ultraflo Max, Depol 740L	Brewers Clarex, Flavourzyme	5,7
C	BSG protein hydrolysate	Depol 740L	Brewers Clarex, Flavourzyme	5,7
D	BSG protein hydrolysate	Shearzyme, Ultraflo Max	Brewers Clarex, Flavourzyme	5,7
E	BSG protein hydrolysate	Shearzyme, Ultraflo Max, Depol 740L	Alcalase, Flavourzyme	5,9,7
F	BSG protein hydrolysate	Depol 740L	Alcalase, Flavourzyme	5,9,7
G	BSG protein hydrolysate	Shearzyme, Ultraflo Max	Alcalase, Flavourzyme	5,9,7
H	BSG protein hydrolysate	Shearzyme, Ultraflo Max, Depol 740L	Alcalase, Brewers Clarex, Flavourzyme	5,9,7
I	BSG protein hydrolysate	Depol 740L	Alcalase, Brewers Clarex, Flavourzyme	5,9,7
J	BSG protein hydrolysate	Shearzyme, Ultraflo Max	Alcalase, Brewers Clarex, Flavourzyme	5,9,7

BSG was incubated with carbohydrases for 4 h at pH5, at 50°C followed by incubation at 50°C with proteases for 4 h. Hydrolysis with Alcalase was carried out a pH9. Hydrolysis with Brewers Clarex and Flavourzyme was carried out at pH7.

Universal 320R centrifuge, Andreas Heittich GmbH & Co., Tuttlingen, Germany) and the resulting supernatant (phenolic-enriched extract) being retained and stored at -20°C prior to analysis. The remaining sediment was incubated at 50°C with Alcalase for 2 h at pH 9 and/or Brewers Clarex/Flavourzyme for 2 h at pH 7. Sediment was then filtered and washed with deionised water at 50°C for 30 minutes. The fibre-enriched sediment was collected and stored at -20°C, while the remaining supernatants were labelled A-J, freeze-dried and stored at -20°C until further analysis.

Cell culture

Jurkat T cells, a human leukemic cell line, were maintained in antibiotic-free Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and plated at a density of 1 x 10^5 and 2 x 10^5 cells mL^{-1} for cell viability and ELISA assays, respectively. RAW 264.7 cells, a murine macrophage cell line were grown in Dulbecco's Modified Eagle Medium (DMEM) media supplemented with 10% FBS. Cells were seeded at a density of 0.2 x 10^5 cells mL^{-1} for cell viability and ELISA assays. U937 cells, a human leukemic monocytic lymphoma cell line were grown in antibiotic-free RPMI-1640 medium supplemented with 10% (v/v) FBS and plated at a density of 1 x 10^5 cells mL^{-1} for the comet assay. HepG2 cells, a human liver carcinoma cell line, were maintained in DMEM supplemented with 10% FBS and plated at a density of 2 x 10^5 cells mL^{-1} for cellular antioxidant assays. Cells were maintained at 37°C in a 5% CO_2 atmosphere. Reduced serum media (2.5% FBS) was used for all experiments.

Cell Viability

The (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to measure the effect of the extracts on cell viability in Jurkat T cells and RAW 264.7 cells. Cells were incubated for 24 h at 37°C with BSG hydrolysates at concentrations of 0 to 0.2% (w/v). The MTT assay kit

(MTT I proliferation kit, Roche Diagnostics, West Sussex, UK) was then used to measure cell viability. Absorbance was determined at 570 nm using a microplate reader (VarioskanFlash, Thermo Scientific, Waltham, MA) and cell viability was calculated as a percentage of the control, untreated cells. An inhibitory concentration-50 (IC_{50}) value, the concentration of a compound that reduces cell viability to 50% of the untreated control cells, was determined for each of the BSG hydrolysates.

Cytokine production

Jurkat T cells were stimulated using 50 mg mL^{-1} concanavalin A (ConA) and incubated for 24 h at 37°C with 0.005% (w/v) BSG hydrolysates. The production of cytokines interleukin-6 (IL-6) and interferon gamma (IFN-γ) was measured by ELISA. RAW 264.7 cells were allowed to adhere overnight. Cells were incubated for a further 24 h at 37°C with 0.001% (w/v) BSG hydrolysates and 0.1 mg mL^{-1} lipopolysaccharide (LPS). The production of cytokines IL-6 and tumor necrosis factor-alpha (TNF-α) was measured by ELISA. Cytokine production was determined using eBioscience ELISA kits (Ready-SET-Go kit purchased from eBioscience, Hatfield, UK). Absorbance was measured at 450 nm on a microplate reader (VarioskanFlash, Thermo Scientific, Waltham, MA) and cytokine production was expressed as percentage of ConA-stimulated Jurkat T cells or LPS-stimulated RAW 264.7 cells.

Comet Assay

U937 cells were seeded in a 12-well plate at a density 1 x 10^5 cells mL^{-1} in RPMI-1640 medium with 2.5% FBS. Cells were incubated for 24 h at 37°C with either BSG hydrolysates (0.2% w/v) or ferulic acid (1 μg mL^{-1}). Previous cytotoxicity studies suggested that 0.2% (w/v) was a safe, non-toxic concentration to use in U937 cells (see Chapter 8). 1 μg mL^{-1} ferulic acid has previously been used in the U937 cell line as a safe and effective antioxidant compound (Crowley *et al*., 2017) and was used a positive control here. Cells were subsequently treated with H_2O_2 (75 μM for 30 minutes). Oxidative DNA damage to the U937 cells was

then assessed using the comet assay adapted from Tice *et al*. (1991). Fifty cells were scored for each slide using a fluorescence microscope (Optiphot-2, Nikon) and the Komet 5.5 image analysis software. Data were expressed as percent tail DNA.

Cellular Antioxidant Assays: Glutathione, Superoxide Dismutase and Catalase

HepG2 cells (2 x 10^5 cells mL^{-1}) were incubated with BSG protein hydrolysates (0.08% w/v) for 24 h at 37°C. Previous cytotoxicity data suggested that 0.08% (w/v) was a safe, non-toxic concentration to use in HepG2 cells (see Chapter 8). Following incubation, cells were exposed to 2 mM H_2O_2 for 120 minutes. Cells were harvested, sonicated and centrifuged (800 rpm, 10 minutes) and the supernatant was collected for the determination of antioxidant enzyme activity.

The glutathione (GSH) content of the cells was measured as described previously (Hissin and Hilf, 1976). The GSH content was measured in cells as an indicator of intracellular non-enzymatic antioxidant defences. Briefly, 100 µL supernatant was mixed with sodium phosphate–ethylenediaminetetraacetic acid buffer (1.8 mL) and *σ*-phthaldialdehyde (0.1 mg). The fluorescence intensity of the samples was determined (Thermo Scientific Varioskan Flash microplate reader) at wavelengths of 360 nm (absorption) and 430 nm (emission). The GSH content of the samples was determined from a standard curve (0–2.5 nmol mL^{-1} GSH).

Cellular superoxide dismutase (SOD) activity was measured using the method previously described by Misra and Fridovich, (1977). Briefly, the cell supernatant was diluted in 0.05 M potassium phosphate buffer (pH 7) and xanthine, xanthine oxidase and cytochrome c were added. The xanthine oxidase system generates superoxide anion which reduces cytochrome c and this reaction is inhibited by SOD. The reduction in cytochrome c was used to determine the activity of SOD present in the samples from a standard curve (0-1µg SOD mL^{-1}). Samples were read at 550 nm at 20 minute intervals for at least 5 readings.

Catalase (CAT) activity was assessed using a modification of the method of Baudhuin *et al*. (1964). H_2O_2 was added to the cell supernatants and following

incubation any remaining H_2O_2 was determined spectrophotometrically at 465 nm. One unit of catalase activity was defined as the amount of catalase required to decompose 1 µmol H_2O_2 per min at pH 7.5 and 25°C.

GSH content and SOD and CAT activities were quantified relative to the protein content as nmol mL^{-1} GSH mg^{-1} protein in cell homogenate and SOD and CAT units mg^{-1} protein, respectively. The protein content of the samples was quantified by the bicinchoninic acid (BCA) protein assay as previously described (Smith *et al*., 1985). Data were expressed as a percentage of untreated control cells.

Statistical Analysis

All data represent the mean ± standard error (SE) of at least three independent experiments. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett's test or Tukey's multiple comparison test as analysed using GraphPad Prism 4 (GraphPad software, La Jolla, CA).

Results

Cell viability

The cytotoxic assessment of the BSG protein hydrolysates was carried out in Jurkat T cells and RAW 264.7 cells (Tables 2 and 3). In Jurkat T cells, the hydrolysates were assessed in the concentration range of 0-0.2% (w/v). Samples A and B reduced cell viability to 0% at concentrations greater than 0.025% and 0.04% (w/v), respectively. Similarly, hydrolysate D demonstrated a toxic effect in Jurkat T cells, reducing cell viability to 0% at concentrations greater than 0.08% (w/v). Hydrolysate I was the least cytotoxic of hydrolysates A-J, with no significant reduction in cell viability. Following these results, BSG protein hydrolysates (A-J) were used at 0.005% (w/v) for immunomodulatory assays in Jurkat T cell.

Table 2: Effect of BSG protein hydrolysates A-J (0-0.2% (w/v)) on cell viability in Jurkat T cells

	Cell proliferation (% of control)									
	A	B	C	D	E	F	G	H	I	J
0.005%	76.9 ± 4.6*	67.0 ± 13.6*	92.9 ± 15.4	88.3 ± 11.0	83.6 ± 11.7	75.2 ± 16.5	83.5 ± 8.0	87.2 ± 12.0	79.6 ± 3.9	67.1 ± 7.2
0.01%	48.5 ± 4.1*	49.1 ± 7.4*	82.5 ± 14.4	71.6 ± 10.6	66.0 ± 11.6	68.9 ± 9.8	62.7 ± 6.8*	51.8 ± 7.4*	80.5 ± 7.1	80.7 ± 11.6
0.02%	5.1 ± 4.4*	27.8 ± 11.8*	79.9 ± 13.6	55.1 ± 13.0*	89.4 ± 12.2	75.9 ± 8.0	68.0 ± 7.0*	47.1 ± 4.8*	95.8 ± 5.1	67.3 ± 6.4
0.025%	7.7 ± 4.4*	18.8 ± 6.4*	85.3 ± 13.8	58.2 ± 11.3*	83.7 ± 10.7	66.2 ± 11.4	64.1 ± 4.6*	38.4 ± 8.3*	39.1 ± 14.3	34.1 ± 7.2*
0.04%	0.0 ± 0.0*	24.7 ± 10.0*	94.2 ± 13.7	23.8 ± 12.8*	117.1 ± 1.3	79.5 ± 9.8	67.5 ± 7.6*	24.2 ± 6.3*	69.1 ± 11.2	52.7 ± 14.0
0.06%	0.0 ± 0.0*	0.0 ± 0.0*	89.4 ± 15.1	9.0 ± 6.2*	77.7 ± 5.6	63.2 ± 9.3	51.9 ± 8.7*	37.7 ± 6.6*	93.2 ± 12.7	74.5 ± 18.2
0.08%	0.0 ± 0.0*	0.0 ± 0.0*	77.0 ± 15.9	2.4 ± 2.4*	88.7 ± 4.2	65.1 ± 0.6	51.8 ± 4.4*	40.3 ± 9.1*	49.1 ± 23.0	31.4 ± 15.7*
0.1%	0.0 ± 0.0*	0.0 ± 0.0*	86.5 ± 13.8	0.0 ± 0.0*	48.9 ± 6.7*	56.0 ± 15.2*	45.9 ± 6.5*	36.2 ± 5.0*	45.4 ± 20.5	20.4 ± 10.6*
0.15%	0.0 ± 0.0*	0.0 ± 0.0*	58.7 ± 14.9	0.0 ± 0.0*	47.3 ± 7.4*	42.6 ± 7.2*	45.6 ± 5.7*	25.2 ± 7.8*	99.0 ± 18.7	45.5 ± 23.3
0.2%	0.0 ± 0.0*	0.0 ± 0.0*	19.3 ± 19.0*	0.0 ± 0.0*	15.3 ± 8.2*	38.6 ± 3.5*	14.7 ± 13.3*	33.2 ± 4.6*	65.9 ± 34.1	58.6 ± 19.9
IC_{50}	0.01	0.01	0.15	0.02	0.12	0.15	0.07	0.02	n/d	0.06

Values are mean ± SE of at least three independent experiments. Statistical analysis was by ANOVA followed by Dunnett's test. * Denotes significant difference ($P<0.05$) in cell viability relative to untreated control Jurkat T cells. n/d: IC_{50} was not determinable for the range of concentrations tested. A is unhydrolysed BSG protein. B is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Brewers Clarex and Flavourzyme. C is BSG protein treated with Depol 740L, Brewers Clarex and Flavourzyme. D is BSG protein treated with Shearzyme, Ultraflo Max, Brewers Clarex and Flavourzyme. E is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Alcalase and Flavourzyme. F is BSG protein treated with Depol 740L, Alcalase and Flavourzyme. G is BSG protein treated with Shearzyme, Ultraflo Max, Alcalase and Flavourzyme. H is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. I is BSG protein treated with Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. J is BSG protein treated with Shearzyme, Ultraflo Max, Alcalase, Brewers Clarex and Flavourzyme.

Table 3: Effect of BSG protein hydrolysates A-J (0-0.02% (w/v)) on cell viability in RAW 264.7 cells

	Cell proliferation (% of control)									
	A	B	C	D	E	F	G	H	I	J
0.0005%	62.4 ± 4.7	74.6 ± 5.5	81.6 ± 6.5	80.2 ± 7.4	81.3 ± 12.1	76.0 ± 18.4	74.5 ± 19.0	97.5 ± 6.0	81.5 ± 12.1	81.9 ± 5.1
0.001%	59.0 ± 4.0*	80.3 ± 4.8	77.6 ± 4.3	68.7 ± 5.6	85.8 ± 16.2	82.2 ± 14.6	75.1 ± 8.0	80.1 ± 8.8	68.8 ± 9.5	70.2 ± 5.0*
0.002%	44.5 ± 7.2*	50.1 ± 14.5*	59.3 ± 10.6*	59.2 ± 6.8*	76.3 ± 25.9	65.7 ± 21.7	58.9 ± 14.8*	71.0 ± 9.8*	65.3 ± 6.7	52.8 ± 7.4*
0.0025%	48.0 ± 1.6*	62.4 ± 3.9*	65.7 ± 5.3*	42.3 ± 11.4*	73.3 ± 21.5	71.8 ± 7.3	63.5 ± 6.5	69.1 ± 4.2*	65.3 ± 4.9	50.5 ± 6.7*
0.004%	74.6 ± 14.0	56.5 ± 5.3*	47.9 ± 6.5*	53.1 ± 9.3*	78.5 ± 18.3	65.9 ± 6.7	60.4 ± 5.3	49.5 ± 4.6*	62.6 ± 3.5*	40.5 ± 1.6*
0.006%	47.6 ± 1.4*	48.6 ± 3.4*	55.0 ± 7.8*	50.6 ± 8.1*	74.0 ± 12.6	62.7 ± 8.8	56.6 ± 4.0*	56.9 ± 1.9*	67.7 ± 18.9	48.0 ± 4.4*
0.008%	72.0 ± 20.2	53.7 ± 9.0*	52.6 ± 6.8*	49.6 ± 7.5*	65.0 ± 18.9	52.1 ± 11.2	49.9 ± 7.8*	50.0 ± 4.6*	61.6 ± 4.8*	48.8 ± 2.9*
0.01%	52.1 ± 1.4*	47.3 ± 3.6*	43.4 ± 7.1*	49.4 ± 7.2*	68.9 ± 9.9	61.1 ± 0.5	55.7 ± 5.1*	53.2 ± 4.5*	52.0 ± 3.3*	47.1 ± 4.3*
0.015%	60.0 ± 16.6	40.7 ± 8.4*	43.6 ± 13.5*	41.6 ± 9.0*	56.8 ± 13.5	47.6 ± 11.0*	45.5 ± 10.7*	46.8 ± 7.1*	56.6 ± 5.6*	47.2 ± 6.5*
0.02%	58.1 ± 8.4*	52.3 ± 3.9*	49.2 ± 5.6*	50.4 ± 7.7*	60.1 ± 3.1	54.9 ± 2.0	56.9 ± 8.8*	49.2 ± 3.2*	51.3 ± 8.7*	28.2 ± 3.4*
IC_{50}	n/d	0.009	0.009	0.007	0.06	0.02	0.02	0.01	0.03	0.005

Values are mean ± SE of at least three independent experiments. Statistical analysis was by ANOVA followed by Dunnett's test. * Denotes significant difference ($P<0.05$) in cell viability relative to untreated control RAW 264.7 cells. n/d: IC_{50} was not determinable for the range of concentrations tested. A is unhydrolysed BSG protein. B is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Brewers Clarex and Flavourzyme. C is BSG protein treated with Depol 740L, Brewers Clarex and Flavourzyme. D is BSG protein treated with Shearzyme, Ultraflo Max, Brewers Clarex and Flavourzyme. E is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Alcalase and Flavourzyme. F is BSG protein treated with Depol 740L, Alcalase and Flavourzyme. G is BSG protein treated with Shearzyme, Ultraflo Max, Alcalase and Flavourzyme. H is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. I is BSG protein treated with Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. J is BSG protein treated with Shearzyme, Ultraflo Max, Alcalase, Brewers Clarex and Flavourzyme.

The effects of BSG protein hydrolysates A-J on RAW 264.7 cell viability were assessed in the concentration range of 0-0.02% (w/v) (Table 3). The lower IC_{50} values in RAW 264.7 cells, compared with Jurkat T cells, demonstrated the greater degree of cytotoxicity of the enzyme-extracted hydrolysates. Hydrolysate J was the most cytotoxic extract, showing a significant reduction in cell viability at concentrations above 0.0005% (w/v). The least cytotoxic fraction was hydrolysate E, with no significant reduction in cell viability across the concentration range tested. Following on from these results BSG protein hydrolysates were used at 0.001% (w/v) (A-J) for immunomodulatory assays in RAW 264.7 cells.

Immunomodulatory potential

ELISA was used to assess the impact of BSG protein hydrolysates on cytokine production in both Jurkat T cells and RAW 264.7 cells (Figures 1-4). Hydrolysates A-J were assessed in ConA-stimulated Jurkat T cells at 0.005% (w/v). Hydrolysate J significantly reduced ConA-stimulated IL-6 production to 66.6% of control. ConA-stimulated IFN-γ production in Jurkat T cells decreased in the presence of both hydrolysate F and J, however these reductions were not significant. In RAW 264.7 cells, LPS was used to stimulate cytokine production. Hydrolysates A-J were assessed at a concentration of 0.001% (w/v). Hydrolysates H and J (58.4% and 61.4%) significantly reduced IL-6 production in RAW 264.7 cells. Also of note was the anti-inflammatory effect of the unhydrolysed fraction, A, which reduced IL-6 production to 66.1%. LPS-stimulated TNF-α was also significantly reduced by extract A to 68.4%.

Antioxidant activity

BSG protein hydrolysates demonstrating anti-inflammatory activity were then selected for analysis for their antioxidant potential in U937 cells using the comet assay and in HepG2 cells using the GSH, SOD and CAT assays (Figures 5-8). Hydrolysates A, I and J protected against H_2O_2-induced DNA damage in U937 cells. None of the hydrolysates assessed had a significant effect on GSH content in H_2O_2-treated HepG2 cells. The SOD activity in H_2O_2-treated HepG2 cells

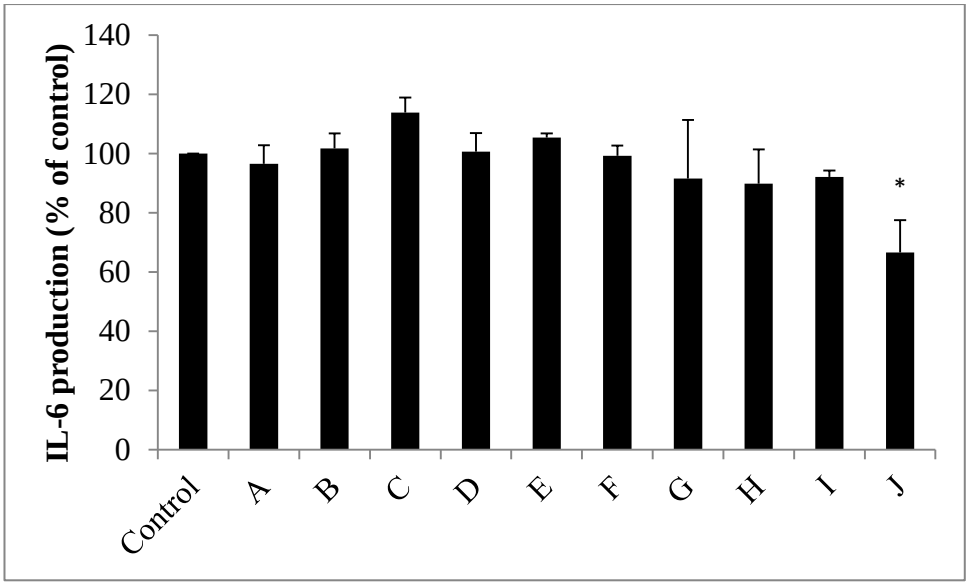


Figure 1: The effect of enzyme-extracted BSG protein hydrolysates A-J (0.005% w/v) on IL-6 production in concanavalin A (ConA)-stimulated Jurkat T cells. Values are a mean ± SE of three independent experiments, expressed as a percentage relative to cells treated with ConA alone. Statistical analysis by ANOVA followed by Tukey's test. * Denotes statistically significant difference in IL-6 production between control and cells treated with protein hydrolysate ($P<0.05$). A is unhydrolysed BSG protein. B is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Brewers Clarex and Flavourzyme. C is BSG protein treated with Depol 740L, Brewers Clarex and Flavourzyme. D is BSG protein treated with Shearzyme, Ultraflo Max, Brewers Clarex and Flavourzyme. E is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Alcalase and Flavourzyme. F is BSG protein treated with Depol 740L, Alcalase and Flavourzyme. G is BSG protein treated with Shearzyme, Ultraflo Max, Alcalase and Flavourzyme. H is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. I is BSG protein treated with Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. J is BSG protein treated with Shearzyme, Ultraflo Max, Alcalase, Brewers Clarex and Flavourzyme.

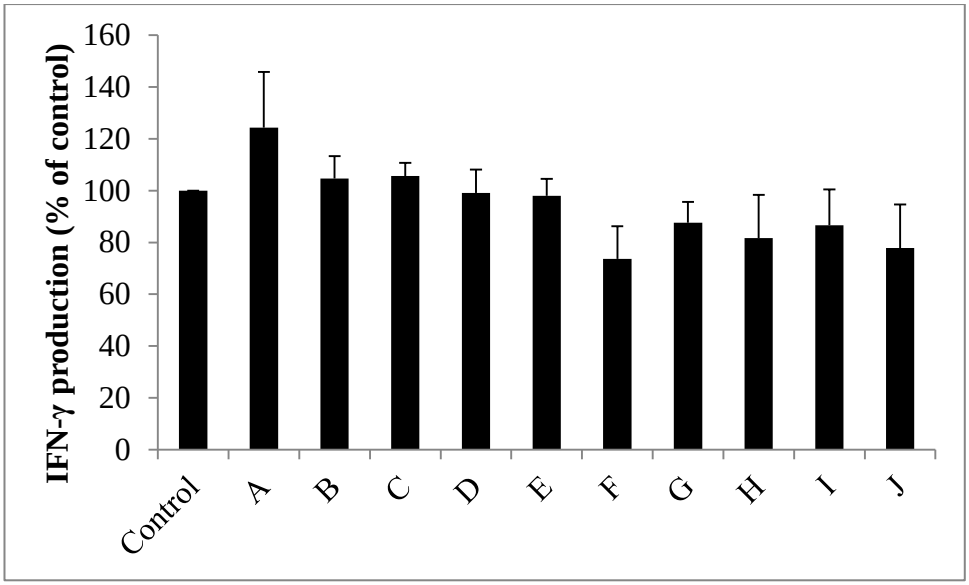


Figure 2: The effect of enzyme-extracted BSG protein hydrolysates A-J (0.005% w/v) on IFN-γ production in concanavalin A (ConA)-stimulated Jurkat T cells. Values are a mean ± SE of three independent experiments, expressed as a percentage relative to cells treated with ConA alone. Statistical analysis by ANOVA followed by Tukey's test. A is unhydrolysed BSG protein. B is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Brewers Clarex and Flavourzyme. C is BSG protein treated with Depol 740L, Brewers Clarex and Flavourzyme. D is BSG protein treated with Shearzyme, Ultraflo Max, Brewers Clarex and Flavourzyme. E is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Alcalase and Flavourzyme. F is BSG protein treated with Depol 740L, Alcalase and Flavourzyme. G is BSG protein treated with Shearzyme, Ultraflo Max, Alcalase and Flavourzyme. H is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. I is BSG protein treated with Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. J is BSG protein treated with Shearzyme, Ultraflo Max, Alcalase, Brewers Clarex and Flavourzyme.

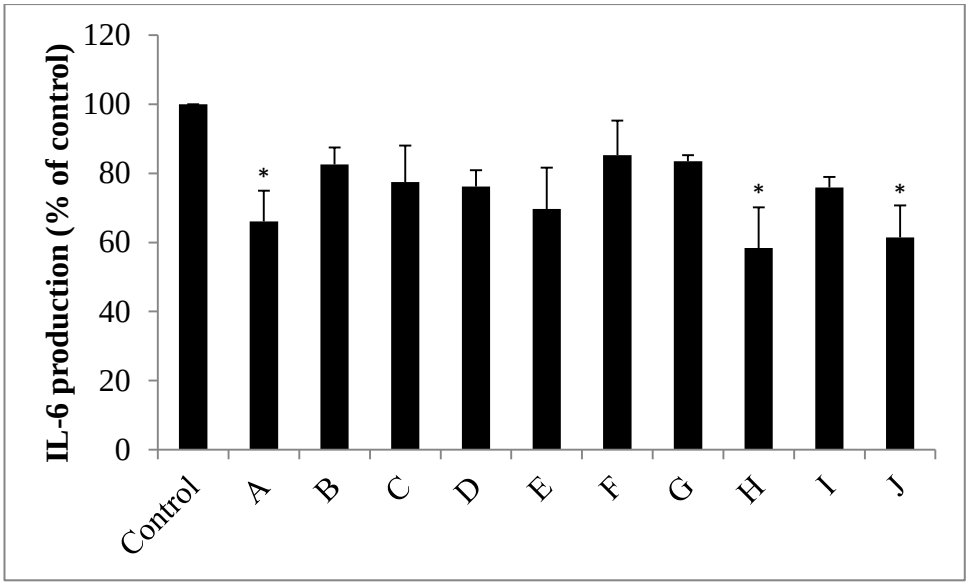


Figure 3: The effect of enzyme-extracted BSG protein hydrolysates A-J (0.001% w/v) on IL-6 production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Values are a mean ± SE of three independent experiments, expressed as a percentage relative to cells treated with LPS alone. Statistical analysis by ANOVA followed by Tukey's test. * Denotes statistically significant difference in IL-6 production between control and cells treated with protein hydrolysate ($P<0.05$). A is unhydrolysed BSG protein. B is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Brewers Clarex and Flavourzyme. C is BSG protein treated with Depol 740L, Brewers Clarex and Flavourzyme. D is BSG protein treated with Shearzyme, Ultraflo Max, Brewers Clarex and Flavourzyme. E is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Alcalase and Flavourzyme. F is BSG protein treated with Depol 740L, Alcalase and Flavourzyme. G is BSG protein treated with Shearzyme, Ultraflo Max, Alcalase and Flavourzyme. H is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. I is BSG protein treated with Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. J is BSG protein treated with Shearzyme, Ultraflo Max, Alcalase, Brewers Clarex and Flavourzyme.

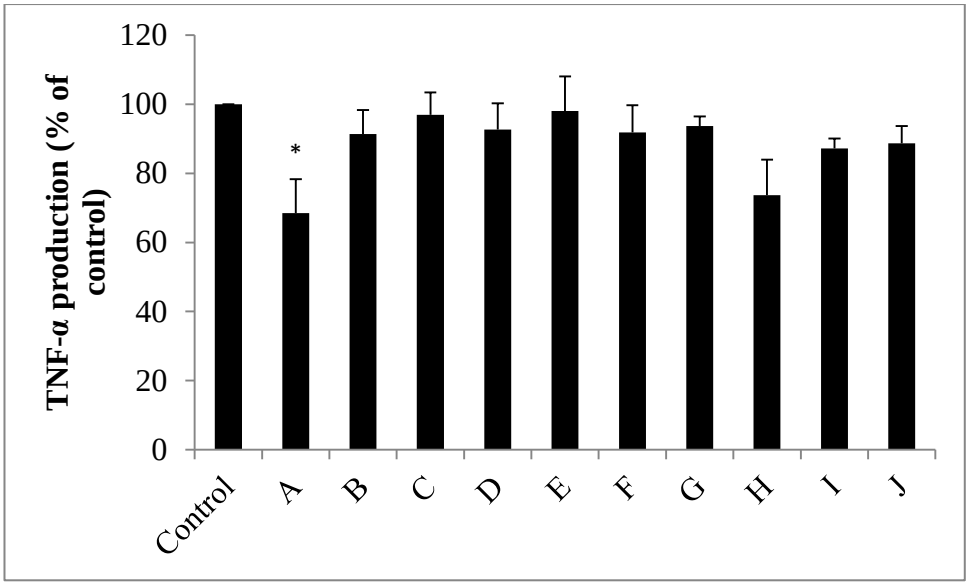


Figure 4: The effect of enzyme-extracted BSG protein hydrolysates A-J (0.001% w/v) on TNF-α production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Values are a mean ± SE of three independent experiments, expressed as a percentage relative to cells treated with LPS alone. Statistical analysis by ANOVA followed by Tukey's test. * Denotes statistically significant difference in TNF-α production between control and cells treated with protein hydrolysate ($P<0.05$). A is unhydrolysed BSG protein. B is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Brewers Clarex and Flavourzyme. C is BSG protein treated with Depol 740L, Brewers Clarex and Flavourzyme. D is BSG protein treated with Shearzyme, Ultraflo Max, Brewers Clarex and Flavourzyme. E is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Alcalase and Flavourzyme. F is BSG protein treated with Depol 740L, Alcalase and Flavourzyme. G is BSG protein treated with Shearzyme, Ultraflo Max, Alcalase and Flavourzyme. H is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. I is BSG protein treated with Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. J is BSG protein treated with Shearzyme, Ultraflo Max, Alcalase, Brewers Clarex and Flavourzyme.

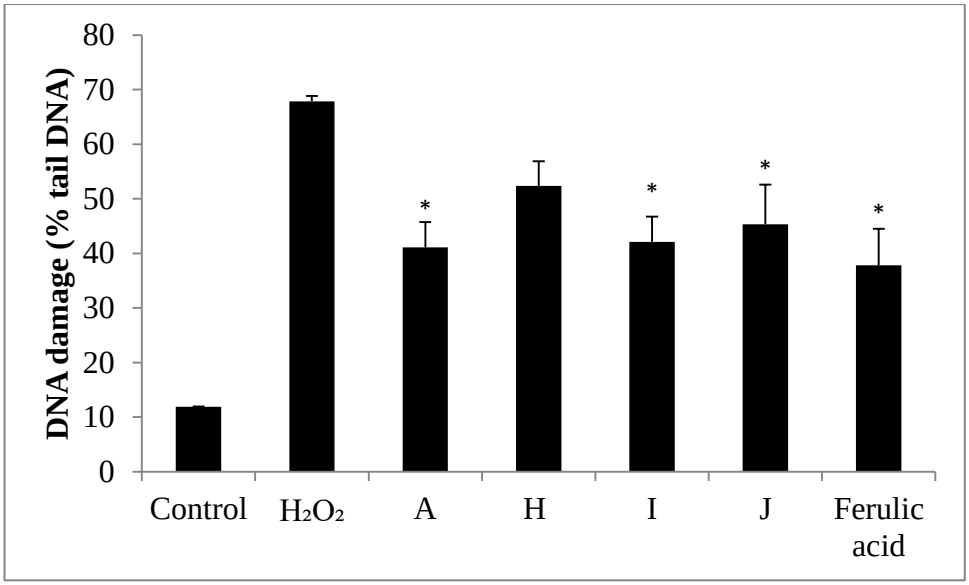


Figure 5: DNA damage in U937 cells treated with 75 μM H_2O_2 for 30 minutes following 24 hour incubation with BSG protein hydrolysates (0.2% w/v) or ferulic acid (1 μg mL^{-1}). Values are mean ± SE of at least three independent experiments. Statistical analysis was by ANOVA followed by Dunnett's test. * Denotes significant difference ($P<0.05$) in DNA damage relative to H_2O_2 control. A is unhydrolysed BSG protein. H is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. I is BSG protein treated with Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. J is BSG protein treated with Shearzyme, Ultraflo Max, Alcalase, Brewers Clarex and Flavourzyme.

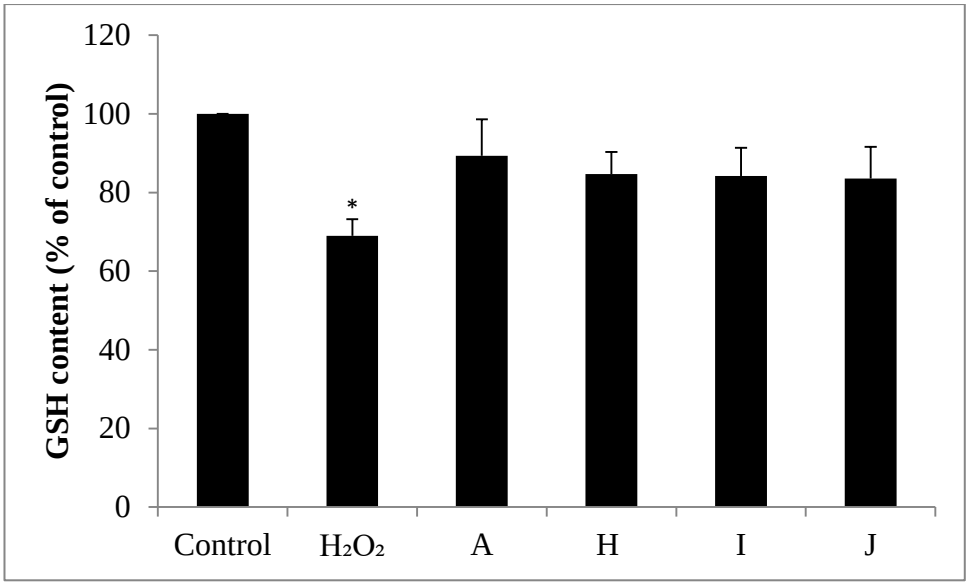


Figure 6: Antioxidant potential of BSG protein hydrolysates (0.08% w/v), measured by their ability to protect against oxidative stress induced by H_2O_2 in the HepG2 cell line, using the glutathione (GSH) assay. Values represent the mean ± SE of at least three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * Statistically significant difference in GSH content between control and H_2O_2 control ($P<0.05$). A is unhydrolysed BSG protein. H is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. I is BSG protein treated with Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. J is BSG protein treated with Shearzyme, Ultraflo Max, Alcalase, Brewers Clarex and Flavourzyme.

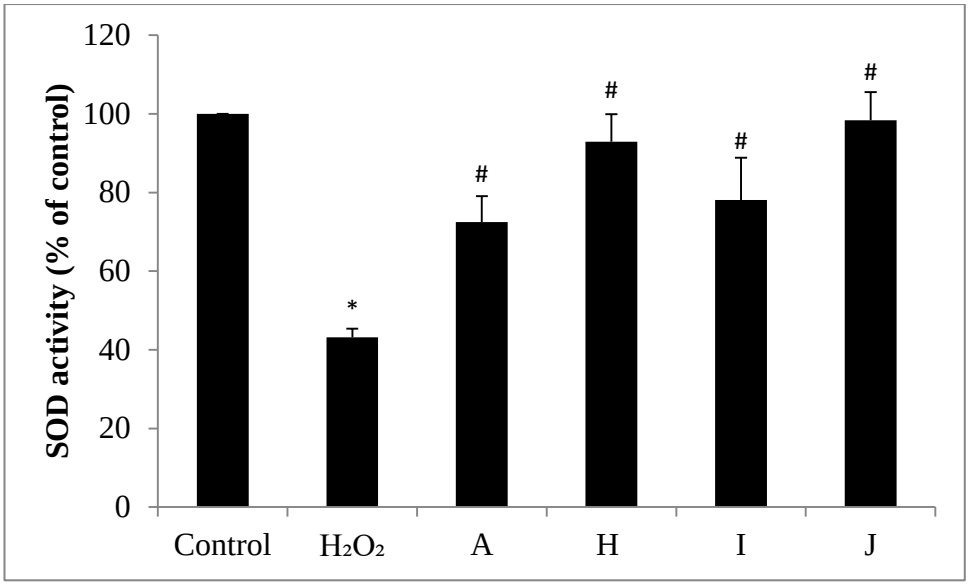


Figure 7: Antioxidant potential of BSG protein hydrolysates (0.08% w/v), measured by their ability to protect against oxidative stress induced by H_2O_2 in the HepG2 cell line, using the superoxide dismutase (SOD) assay. Values represent the mean ± SE of at least three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * Statistically significant difference in SOD activity between control and H_2O_2 control ($P<0.05$). # Statistically significant difference in SOD activity between sample and H_2O_2 control ($P<0.05$). A is unhydrolysed BSG protein. H is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. I is BSG protein treated with Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. J is BSG protein treated with Shearzyme, Ultraflo Max, Alcalase, Brewers Clarex and Flavourzyme.

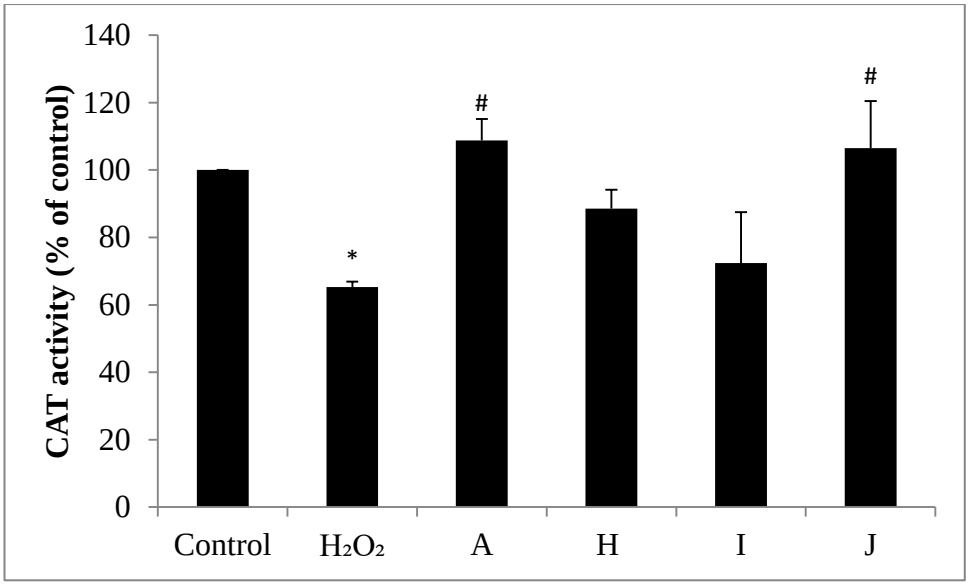


Figure 8: Antioxidant potential of BSG protein extracts (0.08% w/v), measured by their ability to protect against oxidative stress induced by H_2O_2 in the HepG2 cell line, using the catalase (CAT) assay. Values represent the mean ± SE of at least three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * Statistically significant difference in CAT activity between control and H_2O_2 control ($P<0.05$). # Statistically significant difference in CAT activity between sample and H_2O_2 control ($P<0.05$). A is unhydrolysed BSG protein. H is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. I is BSG protein treated with Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. J is BSG protein treated with Shearzyme, Ultraflo Max, Alcalase, Brewers Clarex and Flavourzyme.

was significantly increased when cells were incubated with all BSG protein hydrolysates, including the unhydrolysed fraction, A. Hydrolysate J also exhibited an ability to protect against H_2O_2-induced oxidative stress in HepG2 cells by significantly increasing CAT activity. The unhydrolysed fraction, A also demonstrated cellular antioxidant ability by significantly increasing CAT activity.

Discussion

Research has been conducted on milk proteins as a source of bioactive peptides. However, animal and plant-derived proteins have also shown promise as sources of bioactive peptides (Santiago-Lopez *et al*., 2016). In particular, BSG, a co-product of the brewing industry, has been identified as a source of bioactive peptides. The protein content of BSG is approximately 20% per dry weight basis (Lynch *et al*., 2016). Protein hydrolysates generated from BSG, using an alkaline extraction protocol, have demonstrated anti-inflammatory and antioxidant effects in cell culture models (McCarthy *et al*., 2013a; McCarthy *et al*., 2013b). Enzymatic hydrolysis is an efficient method used for the production of bioactive peptides (Saadi *et al*., 2015). Commercial enzymes such as Alcalase and Flavourzyme have been commonly used for protein hydrolysis (Vieri *et al*., 2017). However, owing to the hemicellulose nature of BSG, enzymes such as xylanase and feruloyl esterases are often required to breakdown this fibrous fraction (Lynch *et al*., 2016). The rigid structure of this material can affect enzyme activity and therefore physical pre-treatment (e.g. shearing) or chemical pre-treatment is often required to allow the polysaccharide structure more open to enzymatic hydrolysis (Lynch *et al*., 2016). The milder conditions used with enzyme hydrolysis means that less damage occurs to the protein substrate, making it a suitable method for the generation of food-grade protein hydrolysates (Power *et al*., 2013). The purpose of this research was to assess the anti-inflammatory and antioxidant effects of BSG protein hydrolysates using an enzyme extraction method.

Nine protein hydrolysates (B-J) were produced from a BSG protein-rich isolate (A), using a combination of carbohydrase (Shearzyme, Ultraflo Max and Depol 740L) and proteinase (Alcalase, Brewers Clarex and Flavourzyme) enzymes. The

protein-rich isolate and associated hydrolysates were assessed with regard to their effects on Jurkat T cell and RAW 264.7 cell viability, cytokine production in Jurkat T cells and RAW 264.7 cells, DNA-protective effects in U937 cells and cellular antioxidant content/enzyme activity in HepG2 cells.

The MTT assay was used to assess cell viability. The assay is a sensitive and cost-effective assay allowing for the high throughput screening of substances that can be carried out in multi-well plates (Cheli and Baldi, 2011). Cell viability can be assessed by exposing cells to a compound of interest, followed by the addition of the MTT (3-(4, 5-dimethylthiazoyl-2-yl)-2, 5- diphenyltetrazolium bromide) substrate. The formation of a formazan product allows for the measure of the number of viable cells and the effect on viability can be quantified in terms of differences in the optical density between treated versus untreated cells (Maestri *et al*., 2016). The BSG samples (A-J) were assessed in Jurkat T cells at concentrations between 0-0.2% (w/v) and in RAW 264.7 cells at concentrations between 0-0.02% (w/v). In Jurkat T cells, samples A and B demonstrated the greatest degree of cytotoxicity, reducing cell viability to 0% of untreated, control cells, at concentrations above 0.025% (w/v) and 0.04% (w/v) respectively (Table 2). Previously, unhydrolysed BSG protein had demonstrated dose-dependent cytotoxic effects in the U937 cell line (McCarthy *et al*., 2013a; McCarthy *et al*., 2013b). The use of the carbohydrase Depol 740L alone to produce hydrolysates C, F and I, demonstrated the least cytotoxic effect as evidenced by the IC_{50} values of these hydrolysates. In RAW 264.7 cells, IC_{50} values were lower indicating that this cell line is more sensitive to the BSG hydrolysates, than Jurkat T cells.

Bioactive compounds often demonstrate an affinity and a specificity for a given receptor typically found in cell membranes, the cytosol or within the nucleus (Serrano *et al*., 2015). Cell culture models can be developed accordingly, allowing for the identification of specific cell subtypes and specific pathophysiological conditions necessary for the bioactive to take effect. This is an important assessment parameter in functional food development (Serrano *et al*., 2015). The immunomodulatory activities of peptides can be assessed by assays measuring the production of cytokines and inflammatory mediators, which are released early in the immune response. Quantifying these signalling molecules can demonstrate the immunostimulatory or immunosuppressive effects of

peptides, firstly, by exposing cells or animals to inflammatory stimuli, treating with peptides and determining the inflammatory response (Maestri *et al*., 2016). Regarding anti-inflammatory activity of bioactive peptides, the modulation of transcription factors, kinases (NF-κB and MAPK) and/or cytosolic compounds are thought to be the primary mechanisms of action (Cicero *et al*., 2017). In the present study, the anti-inflammatory effects of BSG samples were evaluated in ConA-stimulated Jurkat T cells and LPS-stimulated RAW 264.7 cells. In Jurkat T cells, the BSG hydrolysate J, produced using the carbohydrase combination of Shearzyme and Ultraflo Max and the protease combination of Alcalase, Brewers' Clarex and Flavourzyme, significantly reduced ConA-stimulated IL-6 production (Figure 1). Alcalase is a protease from *Bacillus licheniformis*, which has proven to be a very effective with BSG protein solubilisation (Lynch *et al*., 2016). Alkaline-extracted BSG protein hydrolysates produced using Alcalase and Flavourzyme have previously demonstrated an ability to reduce ConA-induced IFN-γ production in Jurkat T cells (McCarthy *et al*., 2013a; McCarthy *et al*., 2013b). Enzyme-extracted BSG samples did not significantly impact on ConA-induced IFN-γ production in Jurkat T cells. Hydrolysates produced using Alcalase, Brewers Clarex and Flavourzyme did demonstrate further anti-inflammatory activity, reducing LPS-stimulated IL-6 production in RAW 264.7 cells (Figure 3). Hydrolysates H and J significantly reduced IL-6 production to 58.4% and 61.4% of the LPS control, respectively. Shearzyme, Ultraflo Max and Depol 740L were the carbohydrases used to produce hydrolysate H. Interestingly, the unhydrolysed fraction A exhibited anti-inflammatory effects in RAW 264.7 cells, significantly decreasing LPS-stimulated IL-6 and TNF-α production in RAW 264.7 cells. McCarthy *et al*. (2013a; 2013b) previously showed that unhydrolysed BSG protein isolates demonstrated an ability to decrease ConA-stimulated IFN-γ production in Jurkat T cells. This suggests that other BSG components may be responsible for the anti-inflammatory activity. However, the individual components of the peptide-rich fractions need to be characterised to find out their mechanisms of action including the specific receptors and signalling pathways involved (Cicero *et al*., 2017). This can help clarify the specific amino acid sequences providing bioactive effects.

Following on from the assessment of the anti-inflammatory activity of BSG protein samples, the cellular antioxidant potential of selected samples was ascertained. The unhydrolysed fraction, A and the hydrolysates produced using Alcalase, Brewers Clarex and Flavourzyme (H-J), were assessed in U937 cells using the comet assay and in HepG2 cells using the GSH, SOD and CAT assays. This approach was recently used to evaluate the antioxidant potential of phenolic compounds from BSG (Crowley *et al*., 2017). Antioxidants can perform at the cell membrane, disrupting peroxyl radical chain reactions, or can react intracellularly with ROS following cellular uptake (Cheli and Baldi, 2011). There is growing evidence from *in vitro* studies of the antioxidant activity of peptides derived from food protein hydrolysates (Power *et al*., 2013). Biomarkers such as cellular antioxidant enzymes and nonenzymatic antioxidants may offer a greater understanding of the specific mechanisms involved in the biological activity of food-derived antioxidant compounds (Cheli and Baldi, 2011). The protective effects of the hydrolysates against oxidant-induced DNA damage was assessed in U937 cells using the comet assay. The protein-rich isolate A and the associated hydrolysates, I and J, demonstrated antioxidant effects, protecting U937 cells against H_2O_2-induced DNA damage (Figure 5). McCarthy *et al*. (2013a; 2013b) showed that alkaline-extracted BSG protein hydrolysates generated using Alcalase and Flavourzyme protected against H_2O_2-induced DNA damage in U937 cells. Recently published results from Vieri *et al*. (2017), showed that three <10kDa BSG protein hydrolysates prepared from brewers' spent yeast proteases, Neutrase and Alcalase, protected against free-radical induced cytotoxicity in both Caco-2 and HepG2 cells. The liver is the principal target for absorbed antioxidant compounds and is also central to xenobiotic metabolism. Accordingly, studies using cultured cells from hepatic origin are widely used to assess the effect of dietary compounds (Cheli and Baldi, 2011). Here, the effect of BSG samples on the GSH content, SOD and CAT activity of HepG2 cells were also investigated. Results showed that all samples had an antioxidant effect protecting against the H_2O_2-induced decrease in SOD activity (Figure 7). The unhydrolysed fraction A and associated hydrolysate J, also protected against H_2O_2-induced decrease in CAT activity (Figure 8).

BSG has been demonstrated as potential source of bioactive peptides. The findings from the present study suggest that use of carbohydrase and proteinase enzymes is an effective way of generating hydrolysates with anti-inflammatory and antioxidant activity. Enzyme hydrolysis procedures are more specific and use milder reaction conditions compared with solvent extraction procedures. The most effective enzyme treatment was seen with the carbohydrase combination of Shearzyme and Ultraflo Max and proteinase combination of Alcalase, Brewers Clarex and Flavourzyme, yielding the BSG hydrolysate J. It is worth noting however, that the enzyme extraction procedure did not enhance the bioactivity of BSG protein, considering that the unhydrolysed fraction A, demonstrated both anti-inflammatory and antioxidant activity. This may indicate that other BSG components may be responsible for the noted bioactivity. More research is required to fully elucidate the molecular mechanisms involved and the specific amino acid sequences that are responsible for the biological activity. There is also a need for animal studies and clinical trials to be conducted in order to uncover any potentially negative effects associated with bioactive peptides (Santiago-Lopez *et al*., 2016). This can lead to the development of more effective functional food products targeting specific health issues.

Conclusions

Both the unhydrolysed fraction and hydrolysate produced using the carbohydrase combination of Shearzyme and Ultraflo Max and proteinase combination of Alcalase, Brewers Clarex and Flavourzyme, demonstrated anti-inflammatory and antioxidant activity. The former suggests that other components present in the BSG may be responsible for the demonstrated bioactivity.

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Chapter 6

Comparison of alkaline and direct enzyme extraction procedures on the generation of brewers' spent grain (BSG) protein hydrolysates with anti-inflammatory and antioxidant potential.

Abstract

Brewers' spent grain (BSG), a cereal grain by-product, is the residual solid fraction left over from brewing, comprising of the husk-pericarp-seed coat portions of the original barley grain. It has been reported to be a potential source of bioactive protein hydrolysates. The cytotoxicity, anti-inflammatory activity and antioxidant potential of BSG protein hydrolysates generated using different extraction procedures were assessed. The extraction methods included alkaline-extraction, a direct enzymatic hydrolysis procedure, alkaline-extracted hydrolysates subjected to simulated gastrointestinal digestion (SGID) and alkaline-extracted hydrolysates prepared using ultrafiltration. The cytotoxicity of BSG protein hydrolysates was determined using the (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The anti-inflammatory activity of the hydrolysates was assessed in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. The impact of the hydrolysates on the glutathione (GSH) content, superoxide dismutase (SOD) and catalase (CAT) activity in hydrogen peroxide (H_2O_2)-treated HepG2 cells was also assessed. Alkaline-extracted BSG protein hydrolysates produced using Prolyve 1000/Protease P and Corolase PP/Flavourzyme, as well as a 10kDa permeate produced using Alcalase/Flavourzyme were effective in reducing LPS-stimulated IL-6 secretion in RAW 264.7 cells. These same protein hydrolysates also displayed antioxidant effects, protecting against H_2O_2-induced decrease in glutathione (GSH) content and H_2O_2-generated decrease in catalase (CAT) activity. All other hydrolysates were not active in any of the bioassays assessed.

Introduction

Cereal grains are considered a staple food in the human diet, contributing an important source of energy. Cereal grains are high in carbohydrates but also contain considerable amounts of protein (Cavazos and Gonzalez de Mejia, 2013). Between 10-15% of the dry grain of cereals constitutes protein (Gangopadhyay *et al*., 2015). Bioactive peptides from cereal grain proteins including from wheat and barley have been reported to display anticancer, antioxidant, antithrombotic,

ACE-inhibitor, DPP-IV inhibitor, and PEP-inhibitor activities (Cavazos and Gonzalez de Mejia, 2013).

Brewers' spent grain (BSG) is an example of a cereal grain by-product and is the residual solid fraction left over from brewing, comprising of the husk-pericarp-seed coat portions of the original barley grain (Mussato *et al*., 2006). It has been reported to be a source of bioactive protein hydrolysates (McCarthy *et al*., 2013a; McCarthy *et al*., 2013b). A hydrolysate is described as a mixture consisting of peptides and amino acids generated through enzyme, acid or alkali treatment or fermentation of the protein (Sarmadi and Ismail, 2010).

Enzyme hydrolysis is a more favourable technique for production of food-grade protein hydrolysates as it utilises milder conditions, resulting in less damage to the protein substrate (Power *et al*., 2013). Most reported biologically active peptides are generated using *in vitro* enzymatic hydrolysis or fermentation (Udenigwe and Aluko, 2012). The simulated gastrointestinal digestion (SGID) procedure mimics the physiological digestion of proteins and allows the assessment of the potential release of biologically active peptides (Udenigwe and Aluko, 2012). Ultrafiltration is a membrane fractionation procedure, involving the separation of proteins/peptides based on molecular weight differences (Power *et al*., 2013). Essentially, a number of factors can impact on the biological activity of peptides including the hydrolytic enzymes employed, processing conditions and peptide size (Udenigwe and Aluko, 2012).

The primary aim of the research was to compare an alkaline extraction method with a direct enzyme extraction procedure, with regard to the generation of BSG protein hydrolysates with anti-inflammatory and antioxidant potential. In addition, the alkaline-extracted BSG protein hydrolysates were subjected to SGID and additionally, alkaline-extracted BSG protein hydrolysates were fractionated using ultrafiltration and all samples were assessed for anti-inflammatory potential.

Materials and Methods

Materials

BSG was obtained from a single batch which was brewed in November 2009, vacuum-packed and stored at -20^{o}C in polypropylene bags until use. Alcalase 2.4L and Flavourzyme® 500L were purchased from Sigma Chemical Co. (Dorset, UK), Corolase® PP was supplied by AB Enzymes (Darmstadt, Germany) and Prolyve 1000 (Pro1k) was kindly provided by Lyven Enzymes Industrielles (Caen, France). RAW 264.7 murine macrophages and HepG2 cells were obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). All other chemicals were purchased from Sigma, unless otherwise stated.

Generation of the alkaline-extracted hydrolysates

The alkaline extracted hydrolysates (44% w/w protein) were prepared as previously described (Connolly *et al*., 2013). The samples was then subjected to hydrolysis using combinations of Alcalase (Alc), Corolase PP (CorPP), Flavourzyme (Flav), Prolyve 1000 (Pro) and/or Protease P (Pro) producing the hydrolysates AlcFla, AlcPro, CorPPFla, and ProPro. Alkaline-extracted BSG protein hydrolysates were labelled A1-A4 (Table 1).

Simulated gastrointestinal digestion (SGID) of alkaline-extracted hydrolysates

SGID of BSG alkaline-extracted hydrolysates was performed according to Walsh *et al*. (2004). Briefly, samples were incubated for 90 min at 37^{o}C, pH 2.0 with pepsin at an E:S of 2.5% (w/w) and then at pH 7.5 with Corolase PP at an E:S of 1.0% (w/w) for a further 150 min. Enzyme inactivation was performed by heating at 80^{o}C for 20 minutes and the samples were subsequently freeze-dried and stored at -20^{o}C. SGID-treated alkaline extracted BSG protein hydrolysates were labelled S1-S4 (Table 1).

Table 1: Summary of methods used to prepare brewers' spent grain (BSG) protein hydrolysates A1-E4.

Sample	Hydrolysis method	Protease enzymes
A1	Alkaline hydrolysis	Prolyve 1000, Protease P
S1	Alkaline hydrolysis followed by SGID	Prolyve 1000, Protease P
E1	Direct enzyme hydrolysis	Prolyve 1000, Protease P
A2	Alkaline hydrolysis	Corolase PP, Flavourzyme
S2	Alkaline hydrolysis followed by SGID	Corolase PP, Flavourzyme
E2	Direct enzyme hydrolysis	Corolase PP, Flavourzyme
A3	Alkaline hydrolysis	Alcalase, Protease P
S3	Alkaline hydrolysis followed by SGID	Alcalase, Protease P
E3	Direct enzyme hydrolysis	Alcalase, Protease P
A4	Alkaline hydrolysis	Alcalase, Flavourzyme
S4	Alkaline hydrolysis followed by SGID	Alcalase, Flavourzyme
E4	Direct enzyme hydrolysis	Alcalase, Flavourzyme

The alkaline extracted extracts were obtained as previously described (Connolly *et al*., 2013). SGID of alkaline-extracted hydrolysates was carried out according to Walsh *et al*. (2004). Enzyme-extracted hydrolysates were generated as outlined in methodology. Extracts were then hydrolysed using combinations of Alcalase, Corolase PP, Flavourzyme, Prolyve 1000 and/or Protease P.

Generation of the enzyme-extracted hydrolysates

The enzyme-extracted hydrolysates were prepared by shearing BSG in distilled H_2O (1:10) at 24,000 rpm for 2 minutes using an Ultra Turrax®T25 high-performance disperser (IKA® Werke GmbH & Co. KG, Staufen, Germany). The BSG suspension was then incubated with carbohydrases: Shearzyme 500 L and Ultraflo Max for 4 h at 50°C. Samples were centrifuged at 2,700 g for 20 minutes at 10°C (Hettich Zentrifugen Universal 320R centrifuge, Andreas Heittich GmbH & Co., Tuttlingen, Germany) and the resulting supernatant (phenolic-enriched extract) being retained and stored at -20°C prior to analysis. The remaining sediment was incubated at 50°C for 4 h with proteases (Alcalase (Alc), Corolase PP (CorPP), Flavourzyme (Flav), Prolyve 1000 (Pro) and/or Protease P (Pro)) producing the hydrolysates AlcFla, AlcPro, CorPPFla, and ProPro. Sediment was then filtered and washed with deionised water at 50°C for 30 minutes. The fibre-enriched sediment was collected and stored at -20°C, while the remaining supernatant were labelled E1-E4, freeze-dried and stored at -20°C until further analysis (Table 1).

Membrane fractionation of alkaline-extracted hydrolysates

Hydrolysates were fractionated using Minimate™ Tangential Flow Filtration Capsules (Pall Corporation, Port Washington, New York, USA). Fractionation was performed using membranes with 30 and 10 kDa nominal molecular mass cut-off values. The three subsequent fractions (30 and 10 kDa permeates and 30 kDa retentate) were freeze-dried and stored at -20^{o}C. Fractionated BSG protein hydrolysates were labelled U1-U6 (Table 2).

Table 2: Summary of methods used to prepare brewers' spent grain (BSG) protein hydrolysates U1-U6.

Sample	Description	Protease enzymes
U1	30kDa Retentate	Corolase PP, Flavourzyme
U2	30kDa Retentate	Alcalase, Flavourzyme
U3	30kDa Permeate	Corolase PP, Flavourzyme
U4	30kDa Permeate	Alcalase, Flavourzyme
U5	10kDa Permeate	Corolase PP, Flavourzyme
U6	10kDa Permeate	Alcalase, Flavourzyme

Extracts were generated using an alkaline hydrolysis method as previously described (Connolly *et al*., 2013). Extracts were then hydrolysed using combinations of Alcalase, Corolase PP and/or Flavourzyme. Fractionation was performed using membranes with 30 and 10 kDa nominal molecular mass cut-off values.

Cell culture

RAW 264.7 cells, a murine macrophage cell line, were grown in Dulbecco's Modified Eagle Medium (DMEM) media supplemented with 10% FBS. Cells were seeded at a density of 0.2 x 10^5 cells mL^{-1} for cell viability and ELISA assays. HepG2 cells, a human liver carcinoma cell line, were maintained in DMEM supplemented with 10% FBS and plated at a density of 2 x 10^5 cells mL^{-1} for cellular antioxidant assays. Cells were maintained at 37°C in a 5% CO_2 atmosphere. Reduced serum media (2.5% FBS) was used for all experiments.

Cell Viability

The (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to measure the effect of the extracts on cell viability in RAW 264.7 cells. Cells were incubated for 24 h at 37°C with BSG protein hydrolysates at concentrations of 0 to 0.02% (w/v). The MTT assay kit (MTT I proliferation kit, Roche Diagnostics, West Sussex, UK) was then used to measure cell viability. Absorbance was determined at 570 nm using a microplate reader (VarioskanFlash, Thermo Scientific, Waltham, MA) and cell viability was calculated as a percentage of the control, untreated cells. An inhibitory concentration-50 (IC_{50}) value, the concentration of a compound that reduces cell viability to 50% of the untreated control cells, was determined for each of the BSG protein hydrolysates.

Cytokine production

RAW 264.7 cells were allowed to adhere overnight. Cells were incubated for a further 24 h at 37°C with 0.01% (w/v) BSG hydrolysates and 0.1 mg mL^{-1} LPS. The production of cytokines interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-α) was measured by ELISA. Cytokine production was determined using eBioscience ELISA kits (Ready-SET-Go kit purchased from eBioscience, Hatfield, UK). Absorbance was measured at 450 nm on a microplate reader

(VarioskanFlash, Thermo Scientific, Waltham, MA) and cytokine production was expressed as percentage of LPS-stimulated RAW 264.7 cells.

Cellular Antioxidant Assays: Glutathione, Superoxide Dismutase and Catalase

HepG2 cells (2 x 10^5 cells mL^{-1}) were incubated with BSG protein hydrolysates (0.08% w/v) for 24 h at 37°C. This concentration was chosen following on from preliminary cytotoxicity data, which noted that 0.08% (w/v) was a safe, non-toxic hydrolysate concentration to use in HepG2 cells (see Table 9, Chapter 8). Following incubation, cells were exposed to 2 mM H_2O_2 for 120 minutes. Cells were harvested, sonicated and centrifuged (800 rpm, 10 minutes) and the supernatant was collected for the determination of antioxidant enzyme activity.

The glutathione (GSH) content of the cells was measured as described previously (Hissin and Hilf, 1976). The GSH content was measured in cells as an indicator of intracellular non-enzymatic antioxidant defences. Briefly, 100 µL supernatant was mixed with sodium phosphate–ethylenediaminetetraacetic acid buffer (1.8 mL) and *σ*-phthaldialdehyde (0.1 mg). The fluorescence intensity of the samples was determined (Thermo Scientific Varioskan Flash microplate reader) at wavelengths of 360 nm (absorption) and 430 nm (emission). The GSH content of the samples was determined from a standard curve (0–2.5 nmol mL^{-1} GSH).

Cellular superoxide dismutase (SOD) activity was measured using the method previously described by Misra and Fridovich, (1977). Briefly, the cell supernatant was diluted in 0.05 M potassium phosphate buffer (pH 7) and xanthine, xanthine oxidase and cytochrome c were added. The xanthine oxidase system generates superoxide anion which reduces cytochrome c and this reaction is inhibited by SOD. The reduction in cytochrome c was used to determine the activity of SOD present in the samples from a standard curve (0-1µg SOD mL^{-1}). Samples were read at 550 nm at 20 minute intervals for at least 5 readings.

Catalase (CAT) activity was assessed using a modification of the method of Baudhuin *et al*. (1964). H_2O_2 was added to the cell supernatants and following incubation any remaining H_2O_2 was determined spectrophotometrically at 465

nm. One unit of catalase activity was defined as the amount of catalase required to decompose 1 µmol H_2O_2 per min at pH 7.5 and 25°C.

GSH content and SOD and CAT activities were quantified relative to the protein content as nmol mL^{-1} GSH mg^{-1} protein in cell homogenate and SOD and CAT units mg^{-1} protein, respectively. The protein content of the samples was quantified by the bicinchoninic acid (BCA) protein assay as previously described (Smith *et al*., 1985). Data were expressed as a percentage of untreated control cells.

Statistical Analysis

All data represent the mean ± standard error (SE) of at least three independent experiments. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett's test or Tukey's multiple comparison test as analysed using GraphPad Prism 4 (GraphPad software, La Jolla, CA).

Results

Cell viability

The BSG protein hydrolysates were assessed at a concentration range of 0-0.02% (w/v). The alkaline-extracted BSG protein hydrolysates A2 (CorPPFla) and A4 (AlcFla) as well as the alkaline-extracted BSG protein hydrolysates subjected to SGID, S2 (CorPPFla) demonstrated significant levels of cytotoxicity in RAW 264.7 cells (Table 3). The incubation of BSG protein hydrolysate A2 led to a dose-dependent effect. Amongst the fractionated hydrolysates, only U1 and U2 showed a significant reduction in cell viability (Table 4). Following on from these results BSG protein hydrolysates were used at 0.01% (w/v) for immunomodulatory assays in RAW 264.7 cells.

Table 3: Effect of BSG protein hydrolysates (0-0.02% (w/v)) on cell viability in RAW 264.7 cells

	Cell proliferation (% of control)											
	A1	S1	E1	A2	S2	E2	A3	S3	E3	A4	S4	E4
0	99.9 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	99.9 ± 0.0	100.0 ± 0.0	99.9 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	99.9 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	99.9 ± 0.0
0.0005	78.6 ± 14.5	79.8 ± 14.2	102.2 ± 6.6	77.2 ± 11.1	69.1 ± 2.7*	66.8 ± 10.9	79.0 ± 12.7	74.0 ± 9.8	88.6 ± 1.8	67.0 ± 5.5*	78.5 ± 3.6	86.0 ± 8.9
0.001	82.2 ± 3.2	80.6 ± 9.8	98.4 ± 7.5	73.0 ± 7.3*	61.8 ± 4.2*	84.7 ± 4.3	76.5 ± 14.8	67.7 ± 10.8	61.4 ± 17.5	59.4 ± 6.7*	73.3 ± 18.3	81.2 ± 11.7
0.002	87.6 ± 9.5	71.5 ± 10.2	78.0 ± 7.8	68.8 ± 6.4*	77.2 ± 9.0	75.1 ± 7.4	72.5 ± 12.3	78.0 ± 18.8	78.3 ± 14.5	58.9 ± 8.0*	66.6 ± 8.3	70.5 ± 12.3
0.0025	78.0 ± 5.9	78.4 ± 9.4	86.5 ± 16.9	64.5 ± 1.9*	66.0 ± 9.0*	82.7 ± 15.7	70.1 ± 12.9	79.6 ± 18.6	85.4 ± 17.5	57.7 ± 5.7*	81.2 ± 25.7	75.2 ± 14.6
0.004	82.8 ± 5.7	87.3 ± 11.1	68.0 ± 6.2	68.1 ± 6.9*	83.5 ± 8.4	70.9 ± 11.5	83.7 ± 18.6	87.4 ± 20.4	72.7 ± 9.0	68.4 ± 11.8*	74.9 ± 11.4	72.8 ± 12.1
0.006	82.2 ± 4.4	85.9 ± 7.4	65.6 ± 16.0	68.9 ± 4.8*	76.7 ± 1.1	74.1 ± 15.0	77.2 ± 13.3	81.6 ± 15.4	72.1 ± 6.4	67.9 ± 8.8*	74.5 ± 18.8	79.3 ± 9.5
0.008	82.4 ± 4.5	78.9 ± 5.8	64.8 ± 13.0	75.7 ± 4.4	76.4 ± 6.5	76.8 ± 14.7	83.2 ± 12.7	80.2 ± 16.5	75.9 ± 9.7	71.0 ± 5.8*	67.2 ± 14.1	81.9 ± 10.8
0.01	81.1 ± 6.9	85.8 ± 7.2	74.5 ± 18.7	68.4 ± 6.0*	72.1 ± 1.7*	81.9 ± 18.5	80.9 ± 11.3	69.9 ± 13.6	74.6 ± 9.4	70.4 ± 4.9*	68.1 ± 17.0	80.8 ± 10.2
0.015	83.3 ± 8.3	78.2 ± 2.3	68.3 ± 18.3	71.6 ± 6.1*	72.6 ± 5.5*	76.0 ± 13.0	83.8 ± 15.6	69.1 ± 11.7	76.0 ± 11.0	64.5± 6.6*	64.2 ± 17.1	80.4 ± 12.4
0.02	78.6 ± 8.5	79.0 ± 5.5	65. 1± 17.6	60.7 ± 3.2*	72.6 ± 6.9*	72.6 ± 16.2	69.0 ± 10.7	64.7 ± 11.8	69.0 ± 12.7	61.0 ± 5.9*	60.0 ± 16.0	79.4 ± 15.3
IC_{50}	n/d	n/d	0.04	n/d	n/d	n/d	n/d	n/d	n/d	n/d	0.32	n/d

Values are mean ± SE of at least three independent experiments. Statistical analysis was by ANOVA followed by Dunnett's test. * Denotes significant difference ($P<0.05$) in cell viability relative to untreated control RAW 264.7 cells. A1 is a BSG protein hydrolysate prepared via alkaline hydrolysis and subjected to further hydrolysis by ProPro. S1 is A1 subjected to SGID. E1 is prepared via direct enzyme hydrolysis by ProPro. A2 is a BSG protein hydrolysate prepared via alkaline hydrolysis and subjected to further hydrolysis by CorPPFla. S2 is A2 subjected to SGID. E2 is prepared via direct enzyme hydrolysis by CorPPFla. A3 is a BSG protein hydrolysate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcPro. S3 is A3 subjected to SGID. E3 is prepared via direct enzyme hydrolysis by AlcPro. A4 is a BSG protein hydrolysate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcFla. S4 is A4 subjected to SGID. E4 is prepared via direct enzyme hydrolysis by AlcFla.

Table 4: Effect of BSG protein hydrolysates (0-0.02% (w/v)) on cell viability in RAW 264.7 cells

	Cell proliferation (% of control)					
	U1	U2	U3	U4	U5	U6
0	99.9 ± 0.0	99.9 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	99.9 ± 0.0	99.9 ± 0.0
0.0005	92.6 ± 6.3	92.7 ± 5.4	110.6 ± 10.4	99.0 ± 5.9	84.0 ± 3.5	77.5 ± 6.9
0.001	73.3 ± 4.7*	79.8 ± 4.5	100.6 ± 3.0	102.4 ± 6.4	84.8 ± 2.5	89.3 ± 4.8
0.002	73.4 ± 1.5*	69.7 ± 7.4*	87.9 ± 5.2	101.8 ± 6.5	89.1 ± 5.3	81.4 ± 0.7
0.0025	76.4 ± 3.5*	77.8 ± 4.4	94.1 ± 3.8	99.4 ± 4.5	75.5 ± 7.2	85.0 ± 6.9
0.004	76.4 ± 6.0*	75.7 ± 2.8	93.8 ± 7.9	93.6 ± 9.7	84.7 ± 13.8	84.5 ± 14.4
0.006	83.1 ± 5.2	83.2 ± 5.1	107.4 ± 8.9	105.8 ± 17.2	93.4 ± 29.9	89.7 ± 15.6
0.008	77.5 ± 3.1	83.3 ± 2.6	97.5 ± 9.2	116.9 ± 21.4	101.8 ± 34.5	101.6 ± 27.2
0.01	82.1 ± 1.5	81.1 ± 3.8	104.4 ± 7.9	129.8 ± 28.0	55.1 ± 15.5	65.7 ± 12.5
0.015	82.3 ± 3.7	85.9 ± 0.6	95.4 ± 8.1	87.5 ± 28.1	42.1 ± 18.0	49.0 ± 19.9
0.02	77.1 ± 12.9*	75.0 ± 16.2	91.7 ± 13.3	82.7 ± 31.5	59.6 ± 28.2	73.1 ± 35.6
IC_{50}	n/d	n/d	0.03	n/d	0.03	n/d

Values are mean ± SE of at least three independent experiments. Statistical analysis was by ANOVA followed by Dunnett's test. * Denotes significant difference ($P<0.05$) in cell viability relative to untreated control RAW 264.7 cells. n/d: IC_{50} was not determinable for the range of concentrations tested. U1 is a 30kDa Retentate prepared via alkaline hydrolysis and subjected to further hydrolysis by CorPPFla. U2 is a 30kDa Retentate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcFla. U3 is a 30kDa Permeate prepared via alkaline hydrolysis and subjected to further hydrolysis by CorPPFla. U4 is a 30kDa Permeate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcFla. U5 is a 10kDa Permeate prepared via alkaline hydrolysis and subjected to further hydrolysis by CorPPFla. U6 is a 10kDa Permeate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcFla.

Immunomodulatory potential

ELISA was used to assess the impact of BSG protein hydrolysates on cytokine production in RAW 264.7 cells (Figures 1-2). LPS was used to stimulate cytokine production. All hydrolysates were evaluated at 0.01% (w/v). Alkaline-extracted hydrolysates A1 and A2 produced using ProPro and CorPPFla respectively, significantly reduced LPS-stimulated IL-6 production in RAW 264.7 cells. The 10kDa permeate, U6, produced using a combination of Alcalase and Flavourzyme, also displayed significant anti-inflammatory activity, reducing LPS-stimulated IL-6 production. These hydrolysates also displayed an ability to reduce LPS-stimulated TNF-α production, although this was not statistically significant.

Antioxidant activity

BSG protein hydrolysates demonstrating anti-inflammatory activity were then selected for analysis for their antioxidant potential in HepG2 cells using the GSH, SOD and CAT assays (Figures 3-5). All BSG protein hydrolysates protected against the H_2O_2-induced decrease in GSH content. None of the hydrolysates assessed had a significant effect on SOD activity in H_2O_2-treated HepG2 cells. The fractionated hydrolysate, U6, significantly restored CAT activity following an oxidative challenge.

Discussion

Including protein hydrolysates in the development of foods has become increasingly popular in the food industry (Saadi *et al*., 2015). Bioactive peptides present in cereals add to their food protein quality value and increase "functionality" of the food as part of the diet (Malaguti *et al*., 2014). Although not as effective as synthetic peptidomimetics and drugs against molecular targets of disease, food-derived peptides are useful owing to their safety, low cost and their advantage of being a source of beneficial and essential amino acids (Udenigwe and Aluko, 2012).

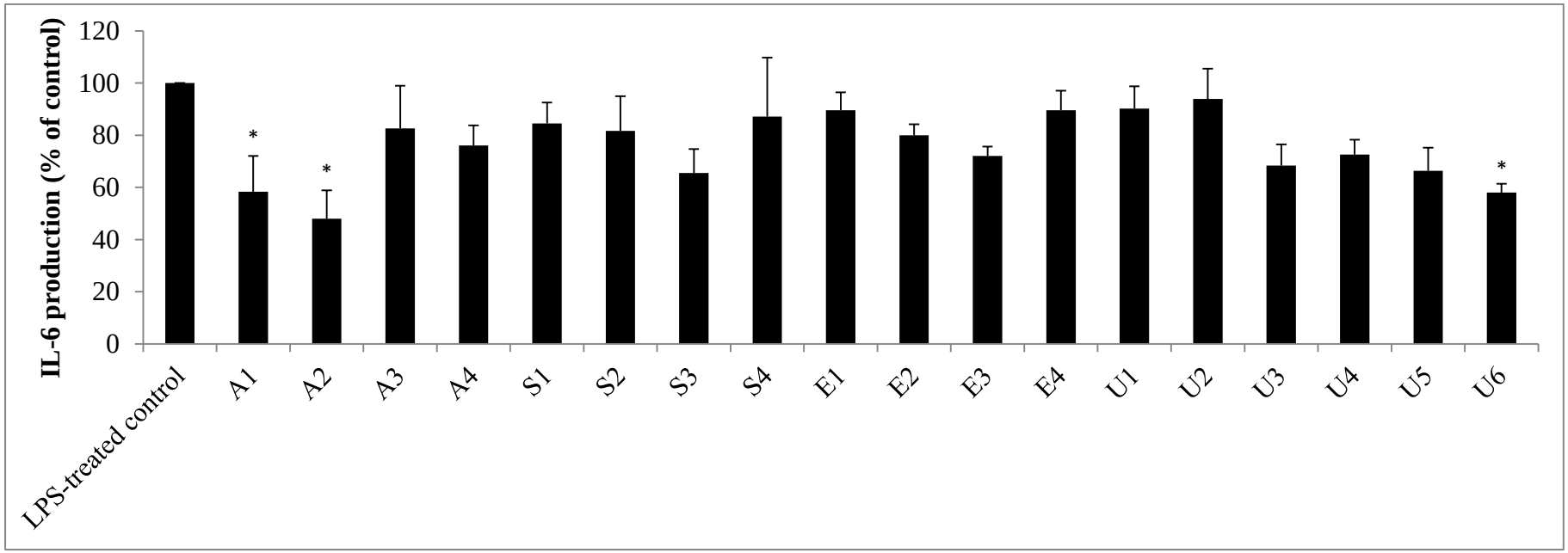


Figure 1: The effect of BSG protein hydrolysates (0.01% w/v) on IL-6 production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. A1-A4 are alkaline-extracted hydrolysates, S1-S4 are alkaline-extracted hydrolysates subjected to SGID, E1-E4 are hydrolysates produced using direct enzyme hydrolysis, U1-U6 are alkaline-extracted hydrolysates produced using ultrafiltration. Values are a mean ± SE of three independent experiments, expressed as a percentage relative to cells treated with LPS alone. Statistical analysis by ANOVA followed by Tukey's test. * Denotes statistically significant difference in IL-6 production between control and cells treated with protein hydrolysate ($P<0.05$).

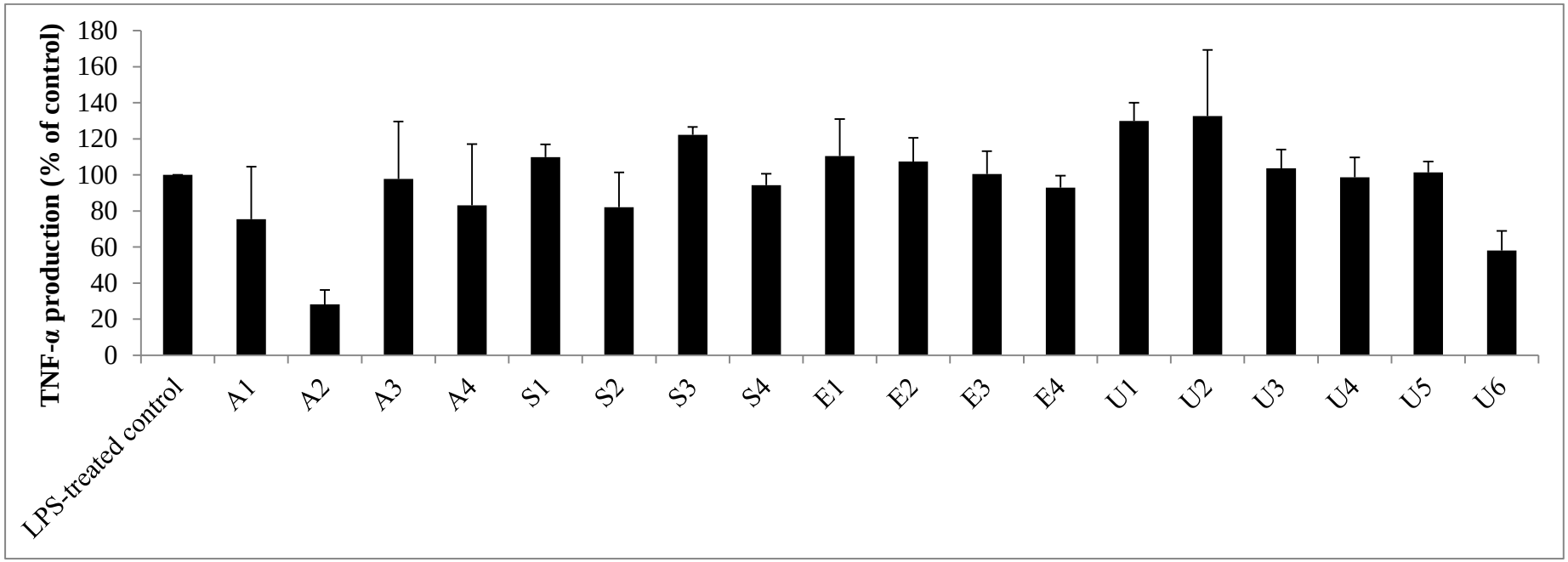


Figure 2: The effect of BSG protein hydrolysates (0.01% w/v) on TNF-α production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. A1-A4 are alkaline-extracted hydrolysates, S1-S4 are alkaline-extracted hydrolysates subjected to SGID, E1-E4 are hydrolysates produced using direct enzyme hydrolysis, U1-U6 are alkaline-extracted hydrolysates produced using ultrafiltration. Values are a mean ± SE of three independent experiments, expressed as a percentage relative to cells treated with LPS alone. Statistical analysis by ANOVA followed by Tukey's test.

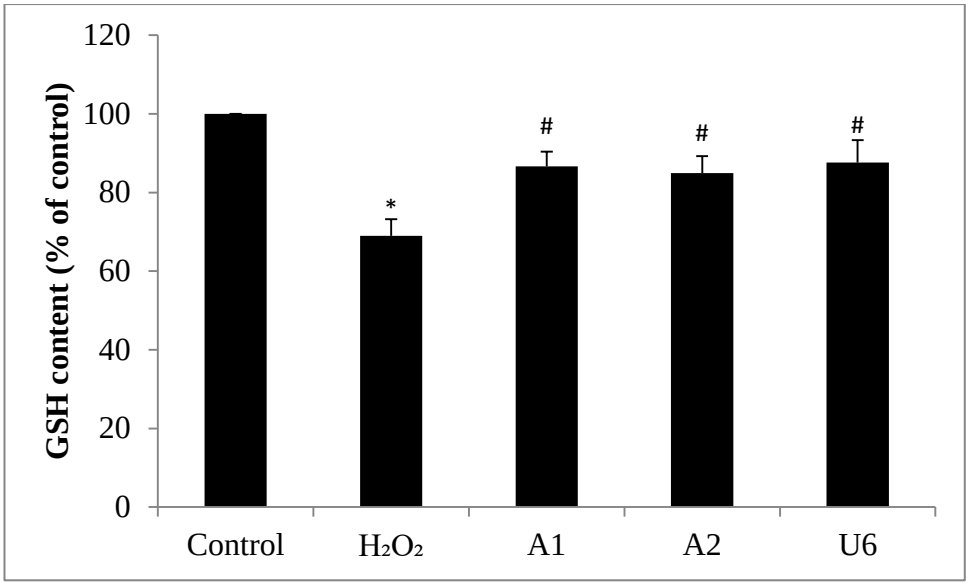


Figure 3: Antioxidant potential of BSG protein hydrolysates (0.08% w/v), measured by their ability to protect against oxidative stress induced by H_2O_2 in the HepG2 cell line, using the glutathione (GSH) assay. Values represent the mean ± SE of at least three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * Statistically significant difference in GSH content between control and H_2O_2 control ($P<0.05$). # Statistically significant difference in CAT activity between sample and H_2O_2 control ($P<0.05$).

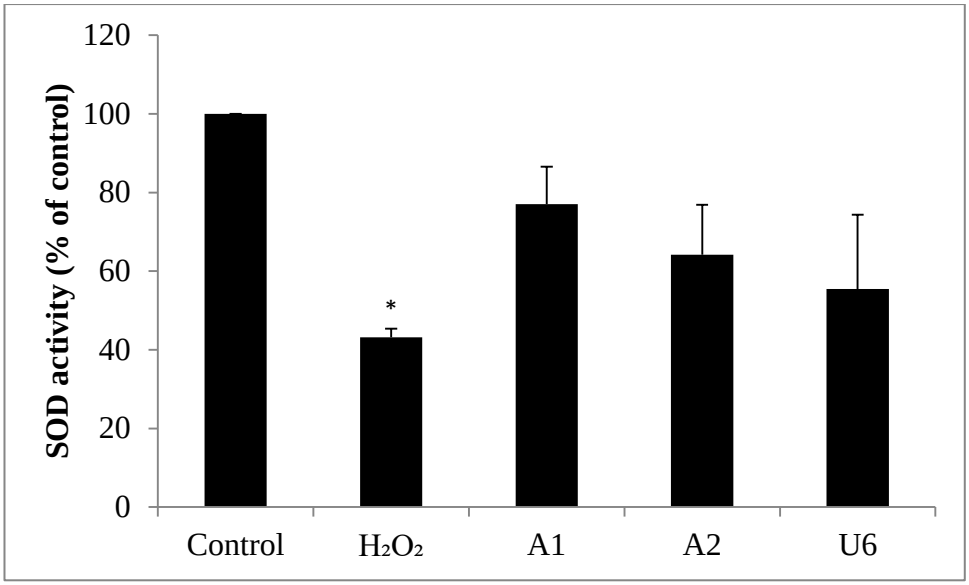


Figure 4: Antioxidant potential of BSG protein hydrolysates (0-0.8% w/v), measured by their ability to protect against oxidative stress induced by H_2O_2 in the HepG2 cell line, using the superoxide dismutase (SOD) assay. Values represent the mean ± SE of at least three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * Statistically significant difference in SOD activity between control and H_2O_2 control ($P<0.05$). # Statistically significant difference in SOD activity between sample and H_2O_2 control ($P<0.05$).

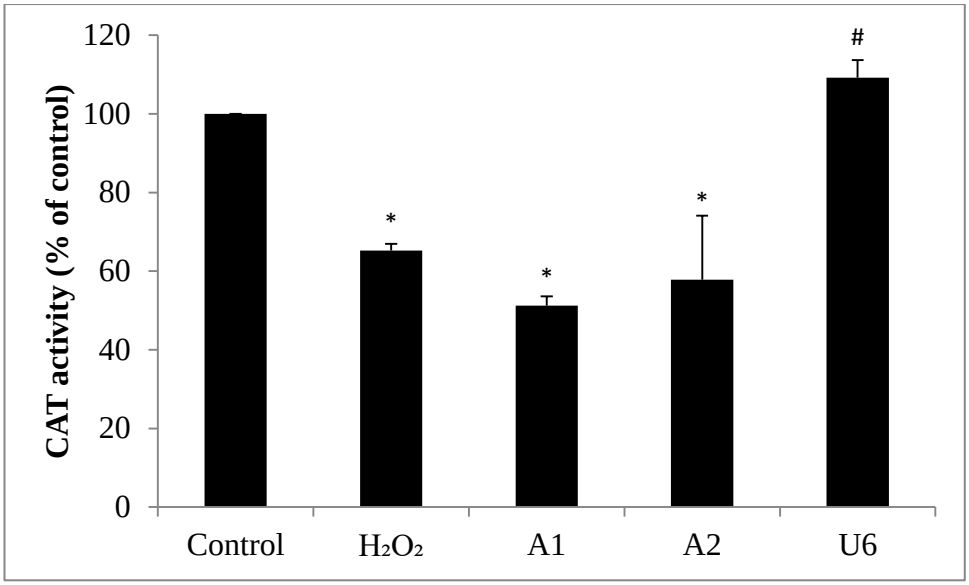


Figure 5: Antioxidant potential of BSG protein extracts (0.08% w/v), measured by their ability to protect against oxidative stress induced by H_2O_2 in the HepG2 cell line, using the catalase (CAT) assay. Values represent the mean ± SE of at least three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. ***** Statistically significant difference in CAT activity between control and H_2O_2 control ($P<0.05$). $^{\#}$ Statistically significant difference in CAT activity between sample and H_2O_2 control ($P<0.05$).

Different processing techniques such as enzyme hydrolysis, alkaline hydrolysis and ultrafiltration have been used to extract the bioactive components of food proteins. Optimization of extraction techniques is necessary for the high yield production of targeted compounds (Saadi *et al*., 2015). Enzymatic hydrolysis is a more favourable process due to its high yield, efficiency and the ability to easily scale it in the laboratory (Saadi *et al*., 2015). Here combinations of Alcalase, Corolase PP, Flavourzyme, Prolyve 1000 and/or Protease P were utilised to generate BSG protein hydrolysates AlcFla, AlcPro, CorPPFla and ProPro. The cytotoxicity, anti-inflammatory activity and antioxidant potential of hydrolysates generated using this direct enzymatic hydrolysis procedure was assessed along with hydrolysates produced using an alkaline-extraction method, alkaline-extracted hydrolysates subjected to SGID and alkaline-extracted hydrolysates generated using ultrafiltration. This work builds on previous research which assessed the bioactivity of BSG protein hydrolysates generated using an alkaline extraction procedure (McCarthy *et al*., 2013a; McCarthy *et al*., 2013b).

The cytotoxicity of BSG protein hydrolysates was measured in RAW 264.7 cells using the MTT assay. The MTT assay has been widely used to assess compound toxicity in a range of different cell lines. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) substrate is added to cultured cells and can be incubated for up to 4 hours. Viable cells that are metabolically active can convert MTT into a purple-coloured formazan product. The amount of formazan can be measured spectrophotometrically measured at 570nm. Dead cells lose the ability to convert MTT into formazan (Riss *et al*., 2013). Here alkaline-extracted BSG protein hydrolysates produced using Corolase PP/Flavourzyme (A2) and Alcalase/Flavourzyme (A4), as well as the alkaline-extracted BSG protein hydrolysate produced using Corolase PP/Flavourzyme and subjected to SGID (S2), demonstrated significant cytotoxicity in RAW 264.7 cells (Table 3). However, even at the highest concentration of the S2 hydrolysate tested (0.02% w/v), cell viability was 72.6% compared to untreated control cells. McCarthy *et al*. (2013a; 2013b) noted that alkaline-extracted BSG protein hydrolysates produced using either Corolase PP, Flavourzyme or Alcalase had a dose-dependent effect on U937 cell viability. Results presented here showed that

the IC_{50} value for some BSG protein hydrolysates could not be determined, suggesting that RAW 264.7 cells have a high tolerance to incubation with this set of BSG protein hydrolysates (Tables 3-4). Karnjanapratum *et al.* (2016) found that, following the assessment of the antiproliferative effects of gelatin hydrolysates from unicorn leatherjacket skin, RAW 264.7 cells demonstrated less cytotoxic effects compared with U937 and HepG2 cells. Also, Udenigwe *et al.* (2009) found that low molecular weight flaxseed peptide fractions did not show toxic effects when assessed in RAW 264.7 cells.

A number of different *in vitro* tests have been used to evaluate the bioactivity of various isolated active compounds, with cell culture models being favoured as a rapid screening tool. Compared to animal studies and clinical trials, cell culture models are easier to manipulate and more reproducible (Saadi *et al*., 2015). As part of this research, the anti-inflammatory activity of BSG protein hydrolysates (0.01% w/v) was assessed in LPS-stimulated RAW 264.7 cells. Immunomodulation concerns the suppression or stimulation of human immune functions (Udenigwe and Aluko, 2012). Cytokines are secreted by different cell types with IL-6, IL-1 and TNF-α being produced during inflammatory states. These cytokines are often targeted for the purpose of therapeutic intervention (Scheller *et al*., 2011). IL-6 is known as being an independent risk factor for coronary artery disease (Kleemann *et al*., 2008). Results presented here showed that in LPS-stimulated RAW 264.7 macrophages, a number of hydrolysates displayed anti-inflammatory activity. Both macrophages and T-lymphocytes generate a multitude of both pro- and anti-inflammatory cytokines (Kleemann *et al*., 2008). Monocytes and macrophages produce TNF-α which displays pro-inflammatory effects in atherosclerosis and other metabolic and inflammatory disorders such as obesity and insulin resistance (Kleemann *et al*., 2008).

Figure 1 shows that alkaline-extracted BSG hydrolysates A1 and A2 displayed IL-6-reducing potential. These hydrolysates were produced using Prolyve 1000/Protease P and Corolase PP/Flavourzyme combinations, respectively. Their direct enzyme hydrolysis equivalents (E1 and E2), did not have any significant impact on LPS-stimulated IL-6 secretion, suggesting that alkaline hydrolysis is a more effective method at producing BSG protein hydrolysates with anti-inflammatory activity. The 10kDa permeate U6 was also effective, reducing IL-6

secretion to 58.1% of the LPS control cells. This hydrolysate was generated via alkaline hydrolysis using Alcalase/Flavourzyme, followed by ultrafiltration. The equivalent BSG protein hydrolysates produced using alkaline hydrolysis (A4) and alkaline hydrolysis followed by SGID (S4), did not significantly decrease IL-6 production, suggesting that, in certain cases, ultrafiltration may be required to elicit an anti-inflammatory effect. Techniques such as membrane ultrafiltration and size-exclusion chromatography are applied to concentrate peptides of set molecular weight ranges, particularly to generate fractions with low molecular weight peptides that can resist further proteolytic digestion *in vivo* (Udenigwe and Aluko, 2012). It has been shown that molecular weight is a variable factor determining the immunomodulatory properties of proteins from different sources (Ko and Jeon, 2015; Kim *et al*., 2016). McCarthy *et al*. (2013b) evaluated the anti-inflammatory activity of alkaline-extracted BSG protein hydrolysates and their accompanying fractionated hydrolysates in ConA-stimulated Jurkat T cells and identified that the >5kDa retentate fractions, as well as unfractionated hydrolysates reduced IFN-γ production to a greater extent than the low molecular weight fractionated samples. Results also presented here showed that there was no significant reduction in LPS-induced IL-6 production when RAW 264.7 cells were incubated with SGID-treated BSG protein hydrolysates. SGID treatments have been utilised previously to evaluate BSG protein hydrolysate bioactivity following addition to foods. Fortification of a snack bar with BSG protein hydrolysates (unfractionated and >5 kDa retentate), which had previously demonstrated anti-inflammatory activity, significantly reduced ConA-stimulated IFN-γ production in Jurkat T cells following *in vitro* digestion (McCarthy *et al*., 2015). Likewise, fortification of a yoghurt drink with the >5 kDa hydrolysate reduced IL-2 secretion, following SGID treatment. Crowley *et al*. (2015) reported that ConA-treated Jurkat T cells had reduced IL-6 secretion when treated with milk fortified with unfractionated and >5 kDa BSG protein hydrolysates and subjected to SGID. The two hydrolysates had previously demonstrated anti-inflammatory activity. The antioxidant and anti-inflammatory effects of cookies baked with different wheat flours, in HepG2 cells following *in vitro* digestion, was assessed by Valli *et al*. (2016). Lower IL-8 production and the higher IL-10 levels were noted in cells supplemented with digested cookies, compared with unsupplemented cells. A multi-compartmental *in vitro* model, simulating the

upper gastrointestinal (GI) tract, was used to digest wheat fractions (Mateo Anson *et al*., 2010). The bioaccessible aleurone fractions were able to reduce LPS-stimulated TNF-α production in U937 macrophages.

None of the BSG protein hydrolysates significantly impacted on TNF-α production. A number of other studies have reported on the anti-inflammatory potential of protein hydrolysates from various cereal grain sources including amaranth (Montoya-Rodriguez *et al*., 2014a; Montoya-Rodriguez *et al*., 2014b; Montoya-Rodriguez and Gonzalez de Meija, 2015), corn gluten (Mochizuki *et al*., 2010) and rice (Boonloh *et al*., 2015). Montoya-Rodriguez *et al*. (2014a) noted that amaranth hydrolysates elicit an anti-inflammatory effect via the inhibition of p65 NF-κB subunit expression in LPS-stimulated in THP-1 macrophages and RAW 264.7 macrophages. In a 2,4,6-trinitrobenzene sulfonic acid-induced rat colitis model, Mochizuki *et al*. (2010) found that the oral administration of an enzymatic hydrolysate of maize gluten (zein) reduced the mucosal levels of inflammatory markers, histamine and myeloperoxidase activity. The oral administration of alkaline or enzyme-extracted rice bran protein hydrolysates to male Sprague-Dawley rats on a high carbohydrate-high fat diet led to a reduction in mRNA expression of proinflammatory cytokines, IL-6, TNF-α, Nos-2 and Mcp-1 in the intra-abdominal fat cells (Boonloh *et al*., 2015). It was proposed that the decrease in these cytokines may be connected to the Ppar-γ upregulation which is involved in fatty acid uptake and glucose metabolism. Other components in the extract enhancing the bioactivity of the hydrolysate fractions were also speculated.

Following on from the assessment of the immunomodulatory ability of BSG protein hydrolysates, the cellular antioxidant potential of the samples was determined. The GSH, SOD and CAT assays were employed to determine the impact of hydrolysates on GSH content and cellular antioxidant activity in HepG2 cells. Oxidative stress can include radical formation, a reduction in antioxidant production, disruption in cellular redox balance and oxidative injury to cellular components, leading to damage to cell membranes, via protein or membrane lipid oxidation or DNA damage (Power *et al*., 2013). Bioactive peptides can exert their antioxidant properties via reactive oxygen species (ROS) scavenging or quenching and the reduction of ROS-triggered oxidation of lipids, proteins and

DNA (Udenigwe and Aluko, 2012). Owing to their anti-inflammatory effects, the hydrolysates A1, A2 and U6 were chosen for analysis of their antioxidant potential. The activity of the enzymes CAT and SOD as well as the content of the antioxidant GSH were also analysed in HepG2 cells (Figures 3-5). All of the hydrolysates significantly protected against the H_2O_2-induced GSH decrease. GSH is classed as a thiol which contains a tripeptide (Cysteine–Glutamic acid–Glycine) and is primarily produced in the liver from the amino substrates (Power *et al*., 2013). None of the hydrolysates protected against the oxidant-induced decrease in SOD activity. The 10kDa permeate produced using Alcalase and Flavourzyme, U6, significantly protected against H_2O_2-generated decrease in CAT activity. SOD and CAT along with glutathione peroxidase (GPx), form part of the antioxidant defence system *in vivo* working in cooperation. The enzymes function by impeding oxidising species and thereby preventing cellular biomolecular damage (Power *et al*., 2013).

It must be noted that *in vitro* assessment of a bioactive compound's activity is not always directly linked to their physiological action *in vivo* owing to issues such as bioavailability, *in vivo* reactivity, *in vivo* stability and tissue storage which cannot be accounted for with *in vitro* systems (Sarmadi and Ismail, 2010). However, owing to their high throughput, reproducibility and relatively low cost, *in vitro* assays are still a useful experimental approach when determining the bioactivity of food compounds.

Conclusions

In conclusion, alkaline hydrolysis was a more effective method than direct enzyme hydrolysis at producing bioactive BSG protein hydrolysates. Both alkaline-extracted BSG protein hydrolysates and alkaline-extracted BSG protein hydrolysates subjected to ultrafiltration, display both antioxidant (GSH content) and anti-inflammatory activity. These BSG fractions may be of potential use for the development of functional food products.

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Chapter 7

Effects of brewers' spent grain (BSG) phenolic extracts on hydrogen peroxide (H_2O_2)-induced apoptosis and lipid peroxidation in SK-N-BE(2) neuronal cells.

Abstract

Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) are often implicated in the neurological damage featured in many neurodegenerative diseases. Antioxidants are thought to elicit both neuroprotective and neuroregenerative effects, via the reduction or reversal of cellular damage and by delaying the advancement of neuronal cell loss. Brewers' spent grain (BSG), a major by-product of the brewing industry, is known as a source of phenolic compounds with cellular antioxidant activity. The neuroprotective potential of BSG phenolic extracts was investigated in SK-N-BE(2) cells treated with H_2O_2. Pale BSG extracts protected against the H_2O_2-induced toxicity in SK-N-BE(2) cells, as assessed by MTT and Neutral Red Uptake (NRU) assays. Extracts from both black and pale BSG significantly decreased the number of apoptotic cells. Black and pale BSG extracts protected against the H_2O_2-induced increase in thiobarbituric acid reactive substances (TBARS). These fractions may have potential as functional food components to prevent and treat neurodegenerative diseases.

Introduction

Neurodegenerative diseases are characterised by the loss of nerve cells from the brain and spinal cord leading to either functional loss (ataxia) or sensory dysfunction (dementia) (Uttara *et al*., 2009). Mitochondrial dysfunction and oxidative stress play a key role in the development of neurodegenerative disorders (Sheikh *et al*., 2013). Oxidative stress can damage biological molecules, resulting in apoptotic or necrotic cell death and eventually leading to neurological damage associated with Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotropic lateral sclerosis (ALS) and cerebral ischemic stroke (CIS). Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), are often implicated in contributing to the damage (Ismail *et al*., 2012; Morelli *et al*., 2014). Evidence suggests that lipid peroxidation is an important mechanism of neurodegeneration in AD (Butterfield and Lauderback, 2002). Unsaturated lipids contained in neuronal cells are susceptible to peroxidation and oxidative modification (Uttara *et al*., 2009). For example, the

brain is rich in polyunsaturated fatty acids (PUFA), such as arachidonic and docosohexanoic acid, which are highly oxidizable (Butterfield *et al*., 2002). The thiobarbituric acid reactive substances (TBARS) assay is used to determine lipid peroxidation (Butterfield and Lauderback, 2002) and is commonly used throughout neurodegenerative research (Fukui *et al*., 2012; Okoko and Ere, 2012).

Biological antioxidants and antioxidant enzymes regulate ROS generation in normal cells (Morelli *et al*., 2014). Antioxidants are thought to elicit both neuroprotective (preventing apoptosis) and neuroregenerative effects, via the reduction or reversal of cellular damage and by delaying the advancement of neuronal cell loss (Pacifico *et al*., 2014). Compounds such as flavonoid polyphenols, non-flavonoid polyphenols, phenolic acids and organosulfur compounds are known for their antioxidant properties (Sultana, 2012). There is evidence to suggest that food-derived antioxidants are useful compounds aimed at reducing neurodegenerative disorders. Hydroxycinnamic acids (HCAs) such as *p*-coumaric, caffeic, ferulic, and sinapic acids are a major class of phenolic acids widely found in tea leaves, coffee, red wine, fruits, vegetables and whole grains cereals. HCAs are powerful antioxidant compounds and are known in particular for their preventive and/or therapeutic role in diseases associated with oxidative stress (e.g., atherosclerosis, inflammatory injury, cancer, and cardiovascular diseases) (Teixeira *et al*., 2013).

The major by-product of the brewing industry is brewers' spent grain (BSG). BSG consists of the seed coat–pericarp–husk layers that encased the original barley grain (Mussatto *et al*., 2014; Lynch *et al*., 2016). BSG is an abundant source of phenolic compounds with antioxidant activity, which can be isolated using various extraction methods, including enzymatic extraction (Mussatto *et al*., 2014). HCAs have been demonstrated as the main phenolic compounds present in BSG, with ferulic acid, in particular, being present in substantial amounts (McCarthy *et al*., 2013). The phenolic component of BSG has previously demonstrated antioxidant activity in cell culture models (McCarthy *et al*., 2012; McCarthy *et al*., 2014; Crowley *et al*., 2017). The purpose of this research was to assess the neuroprotective potential of BSG phenolic extracts using a neuronal cell culture model. The BSG phenolic extracts selected for this research had

previously demonstrated cellular antioxidant activity in U937 cells and HepG2 cells (Crowley *et al*., 2017).

Materials and Methods

Materials

All materials were purchased from Sigma-Aldrich Ireland Limited (Co. Wicklow, Ireland), unless otherwise stated. SK-N-BE(2) cells were purchased from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK).

Preparation of phenolic extracts

The preparation of BSG phenolic extracts was previously described by Crowley *et al*. (2017). The phenolic preparation from black, wet BSG was designated BW2; from black, dry BSG were BD1 and BD2; from pale, dry BSG were PD1, PD2 and PD3 and from pale, wet BSG PW2 and PW3.

Cell culture

SK-N-BE(2) cells, a human neuroblastoma cell line, were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and plated at a seeding density of 0.5×10^{-5}, 1×10^{-5} and 2×10^{-5} cells mL^{-1} for cell viability, TBARS and apoptosis assays, respectively. Cells were grown at 37°C in a 5% CO_2 atmosphere. Reduced serum media (2.5% FBS) was used for all experiments.

Cell viability

The effect of BSG extracts on SK-N-BE(2) cell viability was measured using the MTT and Neutral Red Uptake (NRU) assays. Cells were incubated for 24 hours at 37˚C with extracts at concentrations of 0 to 20% (v/v). The MTT assay kit (MTT I

proliferation kit, Roche Diagnostics, UK) was then used to measure cell viability. The (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was added to cells and left to incubate for 4 hours, before addition of the solubilisation solution. The plates were allowed to incubate overnight and the absorbance was measured at 570 nm using a microplate reader (VarioskanFlash, Thermo Scientific, Waltham, MA). Cell viability was determined as a percentage of the control, untreated cells. The inhibitory concentration-50 (IC_{50}) value, the concentration of a compound that reduces cell viability to 50% of the untreated, control cells, was determined for each of the extracts.

For the NRU assay, cells were again incubated for 24 hours at 37˚C with extracts at concentrations of 0 to 20% (v/v). Media was then removed and the neutral red dye (0.004% w/v) was added. Cells were then incubated for a further 3 hours to allow uptake of the dye. Dye was then removed and cells were washed with PBS. Ice-cold glacial acetic acid (1% v/v) was then added to cells. After 15 minutes, the plate was agitated and the absorbance was measured at 540 nm using a microplate reader. Cell viability was determined as a percentage of the control, untreated cells.

Effects of BSG phenolic extracts on H_2O_2-induced cytotoxicity

The effects of BSG phenolic extracts on H_2O_2-induced toxicity was measured in SK-N-BE(2) cells using the MTT and NRU assays. For the MTT assay, cells were pretreated with either BSG phenolic extracts BW2 and BD1-2 (0.5% v/v) or PD1-3 and PW2-3 (4% v/v) or ferulic acid (2 mM) for 24 hours. Media was then removed and replaced with fresh media containing H_2O_2 (40 µM). Following further 24 hour incubation, the MTT solution was added to cells and left to incubate for 4 hours, before addition of the solubilisation solution. The plates were allowed to incubate overnight and the absorbance was measured at 570 nm using a microplate reader. Cell viability was determined as a percentage of the control, untreated cells.

For the NRU assay, cells were pretreated with either BSG phenolic extracts BW2, BD1-2, PD1-3 and PW2-3 (20% v/v) or ferulic acid (2 mM) for 24 hours.

Media was then removed, replaced with fresh media containing H_2O_2 (20 µM) and incubated for a further 24 hours. Media was then removed and replaced with neutral red dye. Cells were then incubated for a further 3 hours to allow uptake of the dye. Dye was then removed and cells were washed with PBS. Ice-cold glacial acetic acid was then added to cells. After 15 minutes, the plate was agitated and the absorbance was measured at 540 nm using a microplate reader. Cell viability was determined as a percentage of the control, untreated cells.

Determination of apoptosis

The effect of BSG phenolic extract pretreatment on H_2O_2-induced apoptosis was measured by fluorescence microscopy after staining with Hoechst 33342. SK-N-BE(2) cells were incubated with either BSG phenolic extracts BW2 and BD1-2 (0.5% v/v) or PD1-3 and PW2-3 (4% v/v) or ferulic acid (2 mM). After 24 hour incubation, the media was removed and replaced with fresh media containing H_2O_2 (100 µM). Cells were incubated for a further 24 hours and were then harvested by centrifugation (200 *g*, 10 minutes) to form a pellet. Hoechst 33342 stain (5 µg mL^{-1} PBS) was added, and the samples were incubated at 37°C for 30 minutes. Stained samples were placed on a microscope slide and examined under UV light (330–380 nm) using a Nikon fluorescence microscope (200x magnification). A total of 300 cells in each sample were analysed, and the percentage of condensed/fragmented (apoptotic) nuclei was calculated.

Thiobarbituric acid reactive substances (TBARS)

TBARS was measured as an index of lipid peroxidation by a modification of the method of Kornburst & Mavis (1980). SK-N-BE(2) cells were incubated with either BSG phenolic extracts BW2 and BD1-2 (0.5% v/v) or PD1-3 and PW2-3 (4% v/v) or ferulic acid (2 mM). After 24 hour incubation, the media was removed and replaced with fresh media containing H_2O_2 (100 µM). Following 24 hour incubation the media was removed and cells were scraped in a solution of KCl (1.15% w/v). Cells were then pulse sonicated at 13 amps/s and lipid peroxidation was then induced in the samples by the addition of $FeS0_4$ (5 mM)

and ascorbic acid (2 mM) in an 80 mM Tris maleate buffer (pH 7.4). A solution of TBA (0.375% w/v), TCA (15% w/v) and HCl (0.25 N) was added to the samples followed by vortexing and boiling for a period of 15 minutes. Lipid peroxides react with this solution resulting in the formation of a pink fluorochrome (malondialdehyde; MDA) which was determined spectrophotometrically. The solutions were then allowed to cool and were centrifuged (200 g, 10 minutes). The supernatant fraction was removed and the absorbance was measured at 535 nm. The MDA concentrations (nmol MDA mg protein^{-1}) of the samples were calculated using an extinction coefficient of 1.56 x 10^5 M^{-1} cm^{-1} (Beuge & Aust, 1978). TBARS were expressed as percentage of untreated, control cells. The protein content of each sample was determined by the bicinchoninic acid (BCA) assay, as described by Smith *et al*. (1985).

Statistical analysis

All data represent the mean ± standard error (SE) of at least three independent experiments. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Dunnett's test using GraphPad Prism 4 (GraphPad software, La Jolla, CA).

Results

Cell viability

The effects of BSG phenolic extracts on SK-N-BE(2) cell viability was assessed using the MTT and NRU assays (Tables 1-2). As seen with the MTT assay (Table 1), black BSG extracts (BW2, BD1 and BD2) significantly reduced cell viability across the concentration range tested. Similarly, the pale BSG extract PD1 also significantly reduced SK-N-BE(2) cell viability at concentrations above 0.5% (v/v). No cytotoxic effects were noted with the NRU assay (Table 2).

Table 1: Effect of Brewers' spent grain (BSG) phenolic extracts (0-20% (v/v)) on cell viability in SK-N-BE(2) cells as measured by (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

	0.5%	1%	2%	2.5%	4%	6%	8%	10%	15%	20%	IC_{50} (% v/v)
BD1	60.0 ± 4.2*	60.9 ± 4.6*	59.2 ± 5.0*	58.4 ± 4.0*	55.8 ± 4.7*	53.1 ± 2.9*	56.4 ± 4.2*	56.1 ± 4.2*	55.3 ± 2.1*	42.0 ± 4.0*	22.1
BD2	62.3 ± 6.9*	56.4 ± 5.2*	54.1 ± 4.5*	56.0 ± 6.0*	55.1 ± 4.4*	59.9 ± 5.0*	55.8 ± 3.8*	56.0 ± 4.4*	56.0 ± 4.5*	52.8 ± 3.4*	n/d
PD1	81.4 ± 3.1*	76.8 ± 3.3*	78.9 ± 5.6*	81.4 ± 5.5*	80.0 ± 5.4*	83.4 ± 4.7*	74.2 ± 3.3*	83.1 ± 0.3*	75.9 ± 2.4*	75.7 ± 4.3*	n/d
PD2	110.5 ± 12.4	109.7 ± 13.9	115.6 ± 11.4	116.3 ± 8.9	115.2 ± 8.7	117.1 ± 11.8	111.4 ± 10.7	114.5 ± 9.8	113.0 ± 14.5	105.0 ± 9.1	n/d
PD3	116.3 ± 4.9	120.7 ± 5.9	120.4 ± 8.2	108.5 ± 9.9	108.2 ± 9.0	103.5 ± 13.8	106.4 ± 3.7	114.2 ± 8.5	107.2 ± 11.7	98.6 ± 10.0	n/d
BW2	57.7 ± 1.9*	55.0 ± 3.6*	55.5 ± 1.5*	53.1 ± 1.9*	55.3 ± 3.6*	51.1 ± 4.0*	52.4 ± 4.0*	52.4 ± 4.3*	49.4 ± 5.5*	42.2 ± 6.6*	9.2
PW2	97.6 ± 6.9	93.2 ± 9.4	94.5 ± 6.4	90.2 ± 8.3	87.0 ± 9.8	88.3 ± 4.5	84.0 ± 4.8	89.3 ± 17.1	77.5 ± 8.2	70.0 ± 12.8	n/d
PW3	92.0 ± 1.8	88.3 ± 9.3	83.0 ± 6.9	74.4 ± 4.0*	76.7 ± 3.2*	85.7 ± 5.5	78.7 ± 2.4*	86.1 ± 5.4	82.6 ± 5.5	73.3 ± 4.5*	86.6

Values are mean ± SE of three independent experiments, expressed as percentage relative to untreated SK-N-BE(2) cells. BW2 represent the extract from black, wet BSG; BD1-2 represents extracts from black, dry BSG; PD1-3 represents extracts from pale, dry BSG; PW2-3 represents extracts from pale, wet BSG. Statistical analysis by ANOVA followed by Dunnett's Test. * Denotes statistically significant difference in cell viability, relative to untreated SK-N-BE(2) cells ($P<0.05$).

Table 2: Effect of Brewers' spent grain (BSG) phenolic extracts (0-20% (v/v)) on cell viability in SK-N-BE(2) cells as measured by Neutral Red Uptake (NRU) assay

	0.5%	1%	2%	2.5%	4%	6%	8%	10%	15%	20%	IC_{50} (% v/v)
BD1	106.1 ± 15.4	114.6 ± 6.3	122.6 ± 10.7	121.8 ± 4.0	134.3 ± 7.0*	107.9 ± 4.2	112.2 ± 1.5	132.3 ± 11.2	109.4 ± 0.5	90.4 ± 2.6	n/d
BD2	95.9 ± 14.0	103.5 ± 16.2	109.5 ± 4.5	112.9 ± 10.5	118.5 ± 10.2	113.2 ± 11.1	100.1 ± 22.0	107.0 ± 16.5	119.6 ± 14.0	94.0 ± 13.1	n/d
PD1	115.0 ± 10.7	118.2 ± 6.8	104.6 ± 20.3	128.1 ± 9.8	118.2 ± 17.0	118.2 ± 13.9	125.9 ± 3.4	114.4 ± 10.2	95.4 ± 9.8	75.9 ± 17.8	n/d
PD2	86.4 ± 11.2	105.8 ± 6.6	106.7 ± 10.5	108.3 ± 16.0	100.0 ± 14.0	102.9 ± 16.8	105.0 ± 16.6	95.1 ± 14.4	75.4 ± 14.4	73.6 ± 9.2	25.8
PD3	90.4 ± 4.3	92.0 ± 2.0	100.4 ± 16.6	106.6 ± 11.0	116.4 ± 7.1	101.2 ± 5.4	117.5 ± 10.7	107.7 ± 14.4	84.9 ± 19.3	65.7 ± 14.2	n/d
BW2	114.2 ± 8.4	124. 0 ± 1.9	136.6 ± 13.4*	125.1 ± 2.0	138.8 ± 2.4*	137.7 ± 6.0*	129.2 ± 4.3	134.4 ± 4.9	128.2 ± 11.7	77.7 ± 12.2	n/d
PW2	103.9 ± 8.2	103.3 ± 14.2	108.7 ± 7.1	96.6 ± 8.4	73.9 ± 6.6	80.5 ± 8.6	83.9 ± 10.0	74.9 ± 7.3	76.5 ± 17.0	64.5 ± 13.2	37.2
PW3	121.6 ± 12.0	122.0 ± 11.5	132.7 ± 18.1	120.3 ± 8.6	115.9 ± 16.5	111.5 ± 19.4	118.5 ± 16.6	110.0 ± 14.0	82.2 ± 11.8	72.4 ± 3.0	23.4

Values are mean ± SE of three independent experiments, expressed as percentage relative to untreated SK-N-BE(2) cells. BW2 represent the extract from black, wet BSG; BD1-2 represents extracts from black, dry BSG; PD1-3 represents extracts from pale, dry BSG; PW2-3 represents extracts from pale, wet BSG. Statistical analysis by ANOVA followed by Dunnett's Test. * Denotes statistically significant difference in cell viability, relative to untreated SK-N-BE(2) cells ($P<0.05$).

Effects of BSG phenolic extracts on H_2O_2-induced cytotoxicity

The dose-dependent effects of H_2O_2 on cell viability were firstly assessed in SK-N-BE(2) cells (Figure 1-2). At 40µM H_2O_2, cell viability was decreased to 44.8% of untreated SK-N-BE(2) cells, as measured by the MTT assay (Figure 1).

Cell viability was reduced to 50.5% of untreated SK-N-BE(2) cells, when treated with 20µM H_2O_2, as assessed by the NRU assay (Figure 2). As a result of this, 40µM and 20µM H_2O_2 were chosen for further assessment in the MTT and NRU assays, respectively. The protective effect of BSG phenolic extract pretreatment against H_2O_2-induced cytotoxicity was investigated using the MTT and NRU assays (Figures 3-4). At a concentration of 4% (v/v), pale BSG extracts PD1, PD2, PD3 and PW3 protected against the oxidant-induced toxicity in SK-N-BE(2) cells, as assessed by the MTT assay (Figure 3). The positive control, ferulic acid (2 mM) also protected against the H_2O_2-induced reduction in cell viability. BSG phenolic extracts were used at a higher concentration (20% v/v) in the NRU assay (Figure 4). Extracts PD1 and PD2 demonstrated protective effects against the H_2O_2-induced decrease in neutral red uptake. A protective effect was also noted with ferulic acid (2 mM).

Effects of BSG phenolic extracts on H_2O_2-induced apoptosis

The dose-dependent effects of H_2O_2 on apoptosis in SK-N-BE(2) cells was initially assessed at a concentration range of 0-200µM (Figure 5). 100 µM H_2O_2 was subsequently used to induce apoptosis in SK-N-BE(2) cells (Figures 6-7). This led to distinctive cell shrinkage and blebbing, the associated characteristics of apoptosis (Figure 7B). The protective effect of BSG phenolic extract pretreatment was determined by staining cells with Hoechst 33342 and assessing their nuclear morphology using a fluorescent microscope. Extracts BW2 (0.5% v/v), PD2 (4% v/v) and PW2 (4% v/v) significantly decreased the number of apoptotic cells, similar to the positive control, ferulic acid (2 mM) (Figure 7C-F).

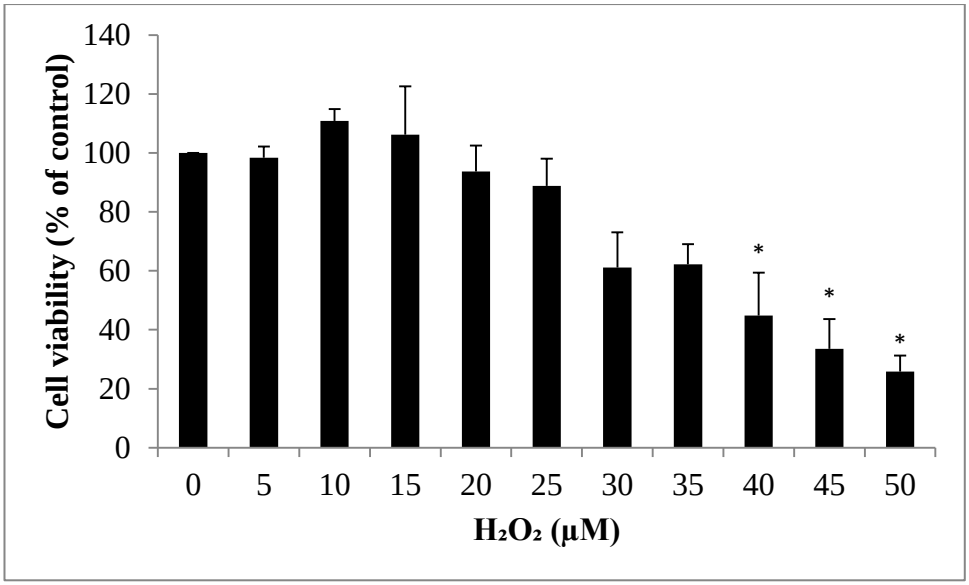


Figure 1: Dose-dependent effects of H_2O_2 (0-50µM) on SK-N-BE(2) cell viability as assessed by (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were treated with H_2O_2 for 24 hours. Values are mean ± SE of at least three independent experiments. Statistical analysis by ANOVA followed by Dunnett's Test. * Denotes statistically significant difference in cell viability, relative to untreated SK-N-BE(2) cells ($P<0.05$).

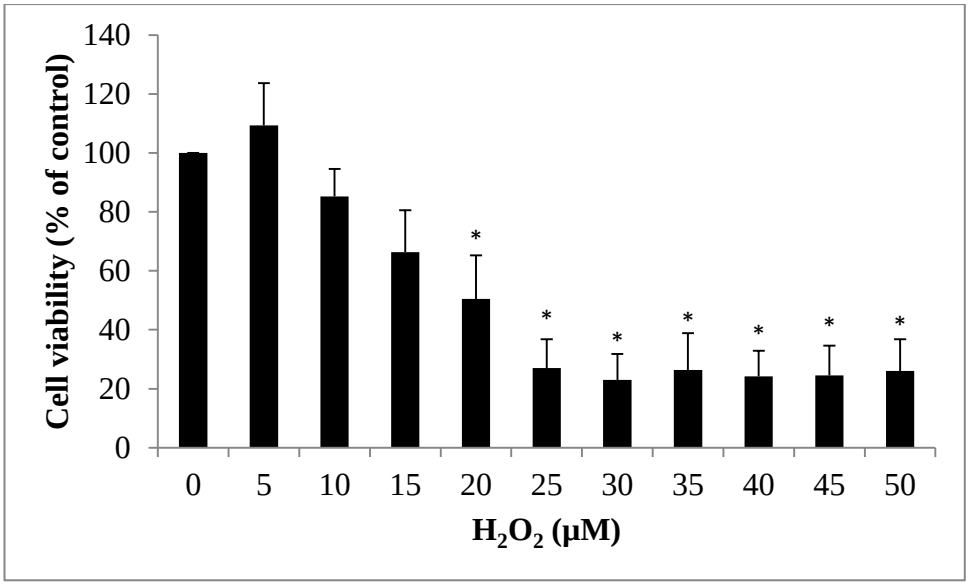


Figure 2: Dose-dependent effects of H_2O_2 (0-50µM) on SK-N-BE(2) cell viability as assessed by NRU assay. Cells were treated with H_2O_2 for 24 hours. Values are mean ± SE of at least three independent experiments. Statistical analysis by ANOVA followed by Dunnett's Test. * Denotes statistically significant difference in cell viability, relative to untreated SK-N-BE(2) cells ($P<0.05$).

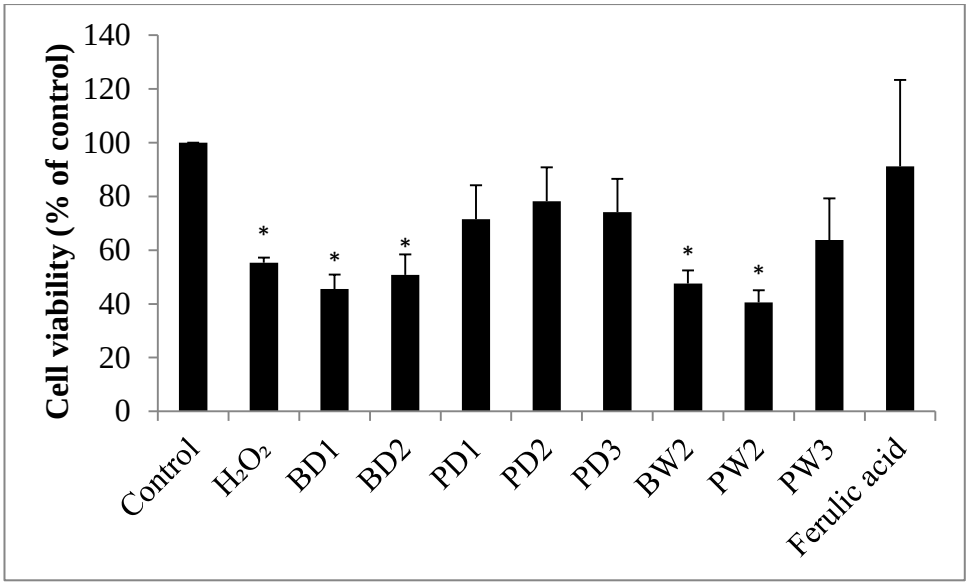


Figure 3: Effect of BSG phenolic extract or ferulic acid pretreatment on SK-N-BE(2) cell viability following 24 hour incubation with 40 µM H_2O_2 as measured by (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were pretreated with BSG extracts BW2, BD1-2 (0.5% v/v), PD1-3, PW2-3 (4% v/v) or ferulic acid (2 mM) for 24 hours, followed by treatment with H_2O_2 for a further 24 hours. Values are mean ± SE of at least three independent experiments. Statistical analysis by ANOVA followed by Dunnett's Test. * Denotes statistically significant difference in cell viability, relative to untreated SK-N-BE(2) cells ($P<0.05$).

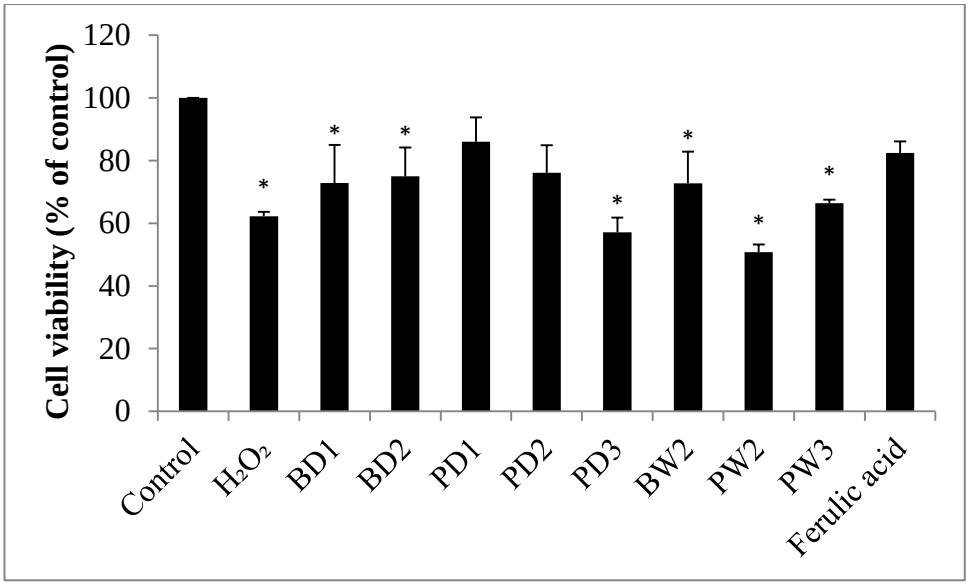


Figure 4: Effect of BSG phenolic extract or ferulic acid pretreatment on SK-N-BE(2) cell viability following 24 hour incubation with 20 µM H_2O_2 as measured by NRU assay. Cells were pretreated with BSG extracts BW2, BD1-2, PD1-3 or PW2-3 (20% v/v) or ferulic acid (2 mM) for 24 hours, followed by treatment with H_2O_2 for a further 24 hours. Values are mean ± SE of at least three independent experiments. Statistical analysis by ANOVA followed by Dunnett's Test. * Denotes statistically significant difference in cell viability, relative to untreated SK-N-BE(2) cells ($P<0.05$).

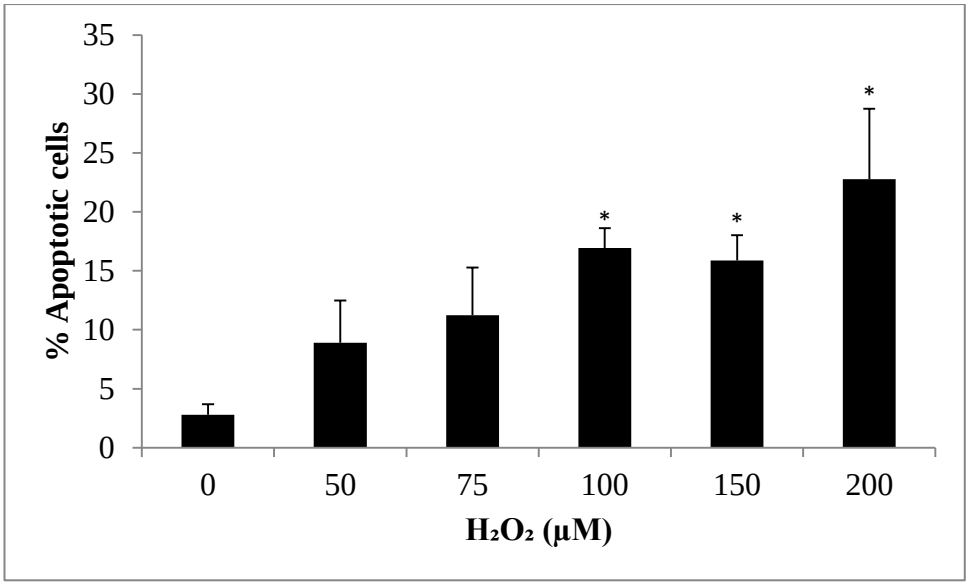


Figure 5: Dose-dependent effects of H_2O_2 (0-200µM) on % apoptosis in SK-N-BE(2) cells. Cells were treated with H_2O_2 for 24 hours. Values are mean ± SE of at least three independent experiments. Statistical analysis by ANOVA followed by Dunnett's Test. * Denotes statistically significant difference in % apoptosis, relative to untreated SK-N-BE(2) cells ($P<0.05$).

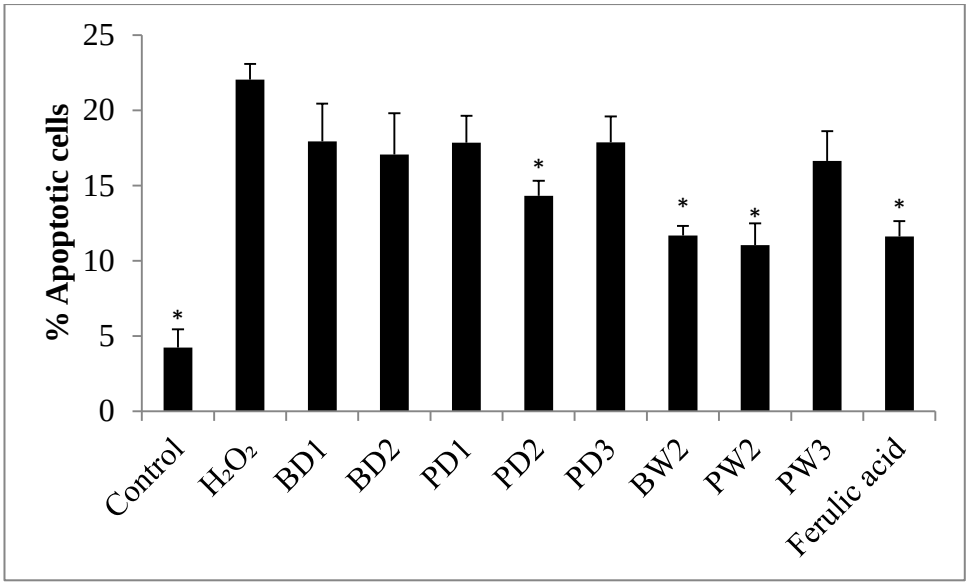


Figure 6: Effect of BSG phenolic extract or ferulic acid pretreatment on H_2O_2-induced apoptosis in SK-N-BE(2) cells. Cells were pretreated with BSG extracts BW2, BD1-2 (0.5% v/v), PD1-3, PW2-3 (4% v/v) or ferulic acid (2 mM) for 24 hours, followed by treatment with 100 µM H_2O_2 for a further 24 hours. Values are mean ± SE of at least three independent experiments. Statistical analysis by ANOVA followed by Dunnett's Test. * Denotes statistically significant difference in cell viability, relative to untreated SK-N-BE(2) cells ($P<0.05$).

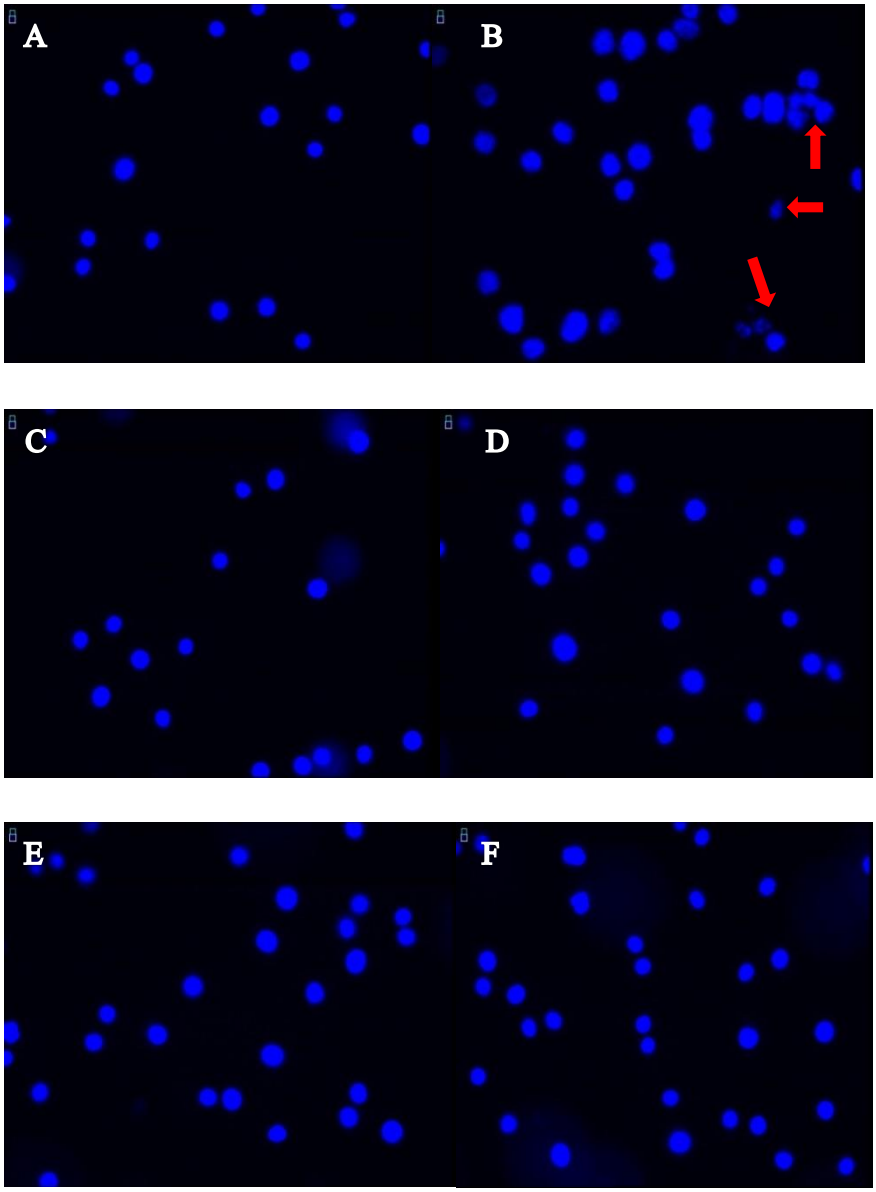


Figure 7: Effect of BSG phenolic extract or ferulic acid pretreatment on cell morphology of H_2O_2-treated SK-N-BE(2) cells. Cells were pretreated with BSG extracts BW2, BD1-2 (0.5% v/v), PD1-3, PW2-3 (4% v/v) or ferulic acid (2 mM) for 24 hours, followed by treatment with 100 µM H_2O_2 for a further 24 hours. Morphological changes of SK-N-BE(2) cells observed by fluorescence microscope after staining with Hoechst 33342. (A) Untreated control cells. (B) Cells treated with H_2O_2 alone, displaying cell shrinkage and blebbing as indicated by red arrows. (C) Cells pretreated with BW2 before H_2O_2 exposure. (D) Cells pretreated with PD2 before H_2O_2 exposure. (E) Cells pretreated with PW2 before H_2O_2 exposure. (F) Cells pretreated with ferulic acid before H_2O_2 exposure.

Effects of BSG phenolic extracts on H_2O_2-induced lipid peroxidation

Lipid peroxidation was induced in SK-N-BE(2) cells by 100 µM H_2O_2 (Figure 8). This led to a statistically significant ($P<0.05$) increase in TBARS compared to the untreated, control cells. The protective effect of BSG phenolic extract pretreatment against oxidant-induced lipid peroxidation was assessed using the TBARS assay. Black BSG extracts (BW2, BD1 and BD2) and the pale extract PD2 protected against the H_2O_2-induced increase in TBARS. A protective effect was also noted with ferulic acid (2mM).

Discussion

Oxidative stress occurs as a result of the change in pro-oxidant/antioxidant homeostasis leading to the production of ROS and free radicals with potentially harmful effects on neuronal cells. ROS production is particularly prevalent in the brain and neuronal tissue (Uttara *et al*., 2009). Brain and neuronal tissue are especially sensitive to oxidative stress (Uttara *et al*., 2009; Sheikh *et al*., 2013), with the brain notably consuming a lot of oxygen, being rich in lipids and having low antioxidant defence activity (Uttara *et al*., 2009).

Various pharmacological compounds are known to have beneficial effects targeted at neurodegenerative disorders. Treatments with natural antioxidants such as polyphenols provide a novel alternative approach by delivering neuroprotective effects through diet or dietary supplements (Albarracin *et al*., 2012). Recently, evidence has emerged on the potential neuroprotective effects of a wide range of food-derived compounds (Chu *et al*., 2009; Vuong *et al*., 2010; Ismail *et al*., 2012). Here, the neuroprotective effects of phenolic extracts from the brewing industry by-product, BSG, were investigated using a neuronal cell culture model. The BSG phenolic extracts chosen had previously demonstrated cellular antioxidant potential protecting against H_2O_2-induced DNA damage in U937 cells and H_2O_2-induced decrease in glutathione (GSH) content, superoxide dismutase (SOD) and catalase (CAT) activity in HepG2 cells (Crowley *et al*., 2017).

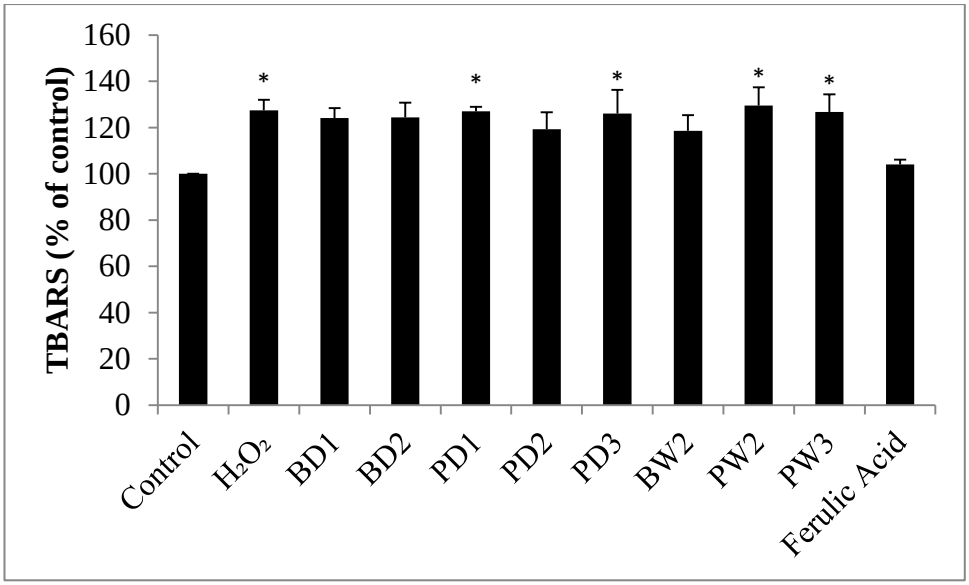


Figure 8: Effect of BSG phenolic extract or ferulic acid pretreatment on H_2O_2-induced lipid peroxidation in SK-N-BE(2) cells. Cells were pretreated with BSG extracts BW2, BD1-2 (0.5% v/v), PD1-3, PW2-3 (4% v/v) or ferulic acid (2 mM) for 24 hours, followed by treatment with 100 µM H_2O_2 for a further 24 hours. Values are mean ± SE of at least three independent experiments. Statistical analysis by ANOVA followed by Dunnett's Test. * Denotes statistically significant difference in cell viability, relative to untreated SK-N-BE(2) cells ($P<0.05$).

The neuroblastoma cell line, SK-N-BE(2) has been widely used in neurodegenerative research (Pacifico *et al*., 2014; Sanchez-Mut *et al*., 2014; Zarrouk *et al*., 2015). Here, SK-N-BE(2) cells were used to study the protective effects of BSG phenolic extracts on H_2O_2-induced toxicity, apoptosis and lipid peroxidation. Firstly, the effects of the BSG phenolic extracts alone on SK-N-BE(2) cell viability was assessed using the MTT and NRU assays. The cells were seeded for 24 hours to allow them to attach to plate wells. Following this, the cells were exposed to BSG phenolic extracts (0-20% v/v) for a further 24 hours. The MTT assay showed that phenolic extracts derived from black BSG significantly reduced cell viability in a dose-dependent manner (Table 1). A similar effect on cell viability of these phenolic extracts was noted in another adherent cell line, HepG2 (Crowley *et al*., 2017). No cytotoxic effects were noted with BSG phenolic extracts, using the NRU assay (Table 2). The two assays measure different aspects of viability. Viability usually describes the percentage of cells which express live cell characteristics such as the uptake/exclusion of a dye. The NRU assay assesses the ability of cells to retain the dye inside the lysosomes with the amount of retained dye being proportional to the number of viable cells. The integrity of the lysosomes, with the accompanying dye binding, is a cell viability indicator (Repetto *et al*., 2008). The MTT assay measures the ability of viable cells with active metabolism to convert MTT into a purple coloured formazan product. Dead cells are unable to convert MTT into formazan, so the colour formation is a useful and convenient marker of viable cells (Riss *et al*., 2013). As a consequence of these results, the protective effects of BSG phenolic extracts against H_2O_2-induced cytotoxicity was assessed using black extracts at; 0.5% (v/v) for MTT assay and 20% (v/v) for NRU assay and pale extracts at; 4% (v/v) for MTT assay and 20% (v/v) for NRU assay.

Following on from this, the protective effects of phenolic extract pretreatment against H_2O_2-stimulated toxicity in SK-N-BE(2) cells was assessed using the MTT and NRU assays. Pretreatment approaches with a number of plant compounds have previously been used to assess the protective effects against H_2O_2-induced cytotoxicity in neuronal cells (Vuong *et al*., 2010; Law *et al*., 2014; Ismail *et al*., 2016). To assess the impact of BSG phenolic extracts on H_2O_2-induced cytotoxicity, SK-N-BE(2) cells were pretreated with black (0.5% v/v for

MTT; 20% v/v for NRU) and pale (4% v/v for MTT; 20% v/v for NRU) extracts for 24 hours, followed by H_2O_2 exposure for a further 24 hours. Wide-ranging concentrations of H_2O_2 have been used to induce toxicity in neuronal cell lines (Chen *et al*., 2009; Morelli *et al*., 2014; Han *et al*., 2014). Here, H_2O_2 concentrations of 40µM and 20µM, induced a 50% reduction in SK-N-BE(2) cell viability, in the MTT and NRU assays, respectively (Figures 1-2). The pale BSG extracts PD1, PD2, PD3 and PW3 all demonstrated a protective effect against H_2O_2-induced cytotoxicity as assessed by the MTT assay. The protective effect of PD3 or PW3 was not seen in the NRU assay however. The protective ability of extracts PD1 and PD2 in both assays suggests that they have an ability to actively reduce the toxic effects caused by H_2O_2 in SK-N-BE(2) cells. Several other studies have demonstrated the protective effects of plant extracts against oxidant-induced toxicity in neuronal cells. Hydroalcoholic extracts of daylily flowers protected against corticosterone- and glutamate-induced decreases in PC12 cell viability, in a dose-dependent manner (Tian *et al*., 2017). Ismail *et al*. (2012) demonstrated that germinated brown rice in H_2O_2-induced toxicity in SH-SY5Y cells. Pretreatment of PC12 and L132 cells with an extract from *Bacopa monniera* demonstrated protective effects against H_2O_2-induced cell death, restoring viability to 85.15 % (PC12 cells) and 83.43 % (L132 cells) of control cells (Pandareesh *et al*., 2016).

Next, the impact of BSG phenolic extract pretreatment on H_2O_2-generated apoptosis was assessed in SK-N-BE(2) cells. As well as mitochondrial dysfunctions and excitotoxicity, apoptosis caused by ROS, such as H_2O_2, can damage biological molecules, resulting in apoptotic or necrotic cell death and subsequently leading to neurodegenerative diseases (Uttara *et al*., 2009; Morelli *et al*., 2014). Many other studies have used H_2O_2 to generate apoptosis in neuronal cells (Chen *et al*., 2009; Han *et al*., 2014; Ismail *et al*., 2016). Here, preliminary experiments demonstrated that 100µM H_2O_2 induced significant levels of apoptosis in SK-N-BE(2) cells (Figure 5). H_2O_2 can induce apoptosis via activation of the mitogen-activated protein kinase (MAPKs) cascades and mitochondrial release of cytochrome c into the cytoplasm (Chen *et al*., 2009; Ismail *et al*., 2012). Subsequent to this, there is a breakdown of mitochondrial membrane potential (MMP) and caspase activation. Morphological change is first

seen as phosphatidylserine (PS) translocation to the outer leaflet of the cell membrane, leading to cell shrinkage, blebbing and DNA fragmentation (Ismail *et al*., 2012). The protective effects of BSG phenolic extracts was assessed by pre-treating cells with extracts (0.5% v/v black BSG, 4% v/v pale BSG) for 24 hours, followed by incubation with 100µM H_2O_2 for a further 24 hours. Treatment of cells with H_2O_2 alone led to increased cell shrinkage and blebbing (Figure 6 and Figure 7B). Pre-treatments of cells with extracts BW2, PD2 and PW2 as well as the positive control, ferulic acid (2mM), significantly protected against H_2O_2-induced apoptosis (Figures 6 and Figure 7C-F). The protective effect of ferulic acid, a major constituent of BSG, has previously been demonstrated. The pre-treatment of HEK293 cells with ferulic acid prior to H_2O_2 exposure protected against the cell shrinkage, chromatin condensation and nuclear fragmentation effects generated by the oxidant (Bian *et al*., 2015). Similarly, ferulic acid inhibited hypoxia-stimulated apoptosis in PC12 cells. The protective effects were thought to occur via the attenuation of ROS generation during hypoxia and the subsequent down-regulation of MAPKs and caspase-3 expression (Lin *et al*., 2015).

A number of different plant-derived compounds have demonstrated protective effects against oxidant-induced apoptosis in neuronal cells. Ismail *et al*. (2012) showed that pre-treating SH-SY5Y cells with germinated brown rice protected against H_2O_2-induced cell death by preventing PS translocation, resulting in the cell membrane staying intact. Another study from the same author (Ismail *et al*., 2016), this time on thymoquinone (bioactive component of black caraway) and a thymoquinone-rich fraction, showed that pre-treating SH-SY5Y cells with both compounds resulted in a reduction H_2O_2-induced apoptosis. The activation of ERK1/2 gene expression by the compounds of action following oxidant insult was speculated as one of its survival mechanisms. Similarly, Chu *et al*. (2009) demonstrated that the protective effects of coffee against H_2O_2-induced primary neuronal cell death may be mediated by modulating the activation of ERK1/2 and JNK1/2 signalling pathways. Fermented blackberry juice protected N2a neuroblastoma cells against H_2O_2-induced oxidative stress via the activation of p38- and JNK-dependent survival pathways and blocking MEK1/2- and ERK1/2-

mediated cell death (Vuong *et al*., 2010). Further research is required to identify the exact neuroprotective mechanisms of BSG phenolic extracts.

Lipid peroxidation plays a role in the etiology of atherosclerosis and neurodegenerative diseases. ROS generated by H_2O_2 can react with glycophospholipids, fatty acids and other essential lipids present in mitochondrial and cell membranes resulting in lipid peroxides and other radicals which can reproduce end products such as acrolein, MDA and 4-hydroxynonenal (HNE) (Butterfield and Lauderback, 2002; Pandareesh *et al*., 2016). The reaction of these compounds with enzymatic, transport or structural proteins, leads to oxidative damage or membrane modification, resulting in the alteration of synaptic membranes and subsequently neuronal cell death (Butterfield and Lauderback, 2002). In the AD brain, lipid peroxidation is extensive. As a therapeutic strategy, antioxidant compounds that can access the brain have the potential to block free radical-induced lipid peroxidation (Butterfield *et al*., 2002). Here the ability of BSG phenolic extract pretreatment to protect against H_2O_2-induced lipid peroxidation was assessed in SK-N-BE(2) cells by the TBARS assay. Results demonstrated that incubation of cells with 100µM H_2O_2 significantly increased the level of lipid peroxidation compared to untreated, control cells (Figure 8). No significant increases in lipid peroxidation were noted following 24 hour pre-incubation with extracts BW2, BD1, BD2 and PD2, suggesting that these fractions contain neuroprotective properties. The positive control, ferulic acid, also demonstrated a protective effect. It has previously been shown that BSG is a rich source of ferulic acid (McCarthy *et al*., 2013). Lin *et al*. (2015) found that ferulic acid significantly inhibited hypoxia-stimulated lipid peroxidation in PC12 cells. Pretreatment of cortical neuronal cells with ferulic acid ethyl ester significantly attenuated amyloid β-peptide (1–42)-induced lipid peroxidation (Sultana *et al*., 2005). One of the disadvantages of the TBARS assay is the reaction of non-lipid molecules with thiobarbituric acid and thus it is therefore considered a nonspecific marker of membrane lipid peroxidation (Butterfield and Lauderback, 2002). It has however been widely used throughout neurodegenerative research (Fukui *et al*., 2012; Pandareesh *et al*., 2016).

BSG phenolic extracts have previously demonstrated antioxidant potential in U937 cells and HepG2, protecting against H_2O_2-induced DNA damage and H_2O_2-

induced decreases in cellular antioxidant content and activity (Crowley *et al*., 2017). Here, SK-N-BE(2) cells were pretreated with BSG phenolic extracts before being exposed to H_2O_2 and the subsequent effects on cell viability, apoptosis and lipid peroxidation were measured. Extracts BW2, PD1 and PD2 all demonstrated significant neuroprotective effects, suggesting that these fractions may have potential as functional food components to prevent and treat neurodegenerative diseases. However, further research is required to identify the exact components eliciting bioactivity and the specific molecular mechanisms that are involved.

Conclusions

To conclude, extracts from both black and pale BSG demonstrated significant neuroprotective effects, indicating that these fractions may be useful as functional food ingredients to prevent and treat neurodegenerative diseases. Future research should focus on identifying the specific components eliciting bioactivity and the molecular pathways that are involved.

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Chapter 8

The inhibitory potential of phenolic extracts and protein hydrolysates derived from brewers' spent grain (BSG) on proliferation of cancer cell lines in culture.

Abstract

Many treatments are effective at treating and controlling cancer, however some adverse side effects can occur, in particular, with chemotherapy. Plant-derived products represent an alternative form of treatment with lesser side effects. Brewers' spent grain (BSG) is known as a source of hydroxycinnamic acids, which are an important group of antioxidant compounds, but these compounds are also known to have inhibitory activity in the proliferation of cancer cell lines. The impact of BSG phenolic extracts and protein hydrolysates on cell proliferation was assessed in the cell lines; Jurkat T cells, RAW 264.7 cells, MCF-7 cells, HepG2 cells and U937 cells. Inhibitory concentrations (IC_{50}) could be calculated for more of the phenolic extracts than for the protein hydrolysates, indicating their greater cytotoxic effects. The phenolic extract from pale, dry BSG (PD3) demonstrated a proapoptotic effect in U937 cells increasing the formation of apoptotic cells and DNA fragmentation. To conclude, BSG phenolic extracts demonstrated greater antiproliferative ability than BSG protein hydrolysates. However, the apoptotic effects of BSG phenolic extracts were, in general, found to be low.

Introduction

Cancer is a multi-stage disease resulting from environmental, chemical, physical, metabolic, and genetic factors involved in the induction and progression of the disease in either a direct and/or indirect manner. The stages involved include initiation, promotion, progression, invasion and metastasis. The promotion of a tumor consists of the active proliferation of cells forming a multi-cellular premalignant tumor cell population (Dai and Mumper, 2010). Apoptosis is a form of cell death which is regulated by a variety of cellular signalling pathways. It is characterised by nuclear condensation and fragmentation, cleavage of chromosomal DNA into fragments and the formation of apoptotic bodies. Tumors can carry mutations which inactivate apoptotic pathways leading to the persistence of tumor cells (Edinger and Thompson, 2004).

Many treatments are effective at treating and controlling cancer, however some adverse side effects can occur, in particular, with chemotherapy. Plant-derived products represent an alternative form of treatment with lesser side effects (Ahmed *et al*., 2016). For example, it is recognised that fruit and vegetable consumption is associated with a reduced cancer risk, owing to its high polyphenol content (Roleira *et al*., 2015). Compounds such as phenolics have the potential to curb tumor formation and growth by inducing cell cycle arrest and apoptosis (Dai and Mumper, 2010). Phenolic compounds are ubiquitous in the plant kingdom and are one of the most important groups of secondary metabolites of plants. They contain at least one aromatic ring with one or more hydroxyl groups and their established antioxidant activity owes to their ability to chelate metals by the referred groups. The anticancer activity of phenolic compounds is generally associated with phenolic antioxidant content (Roleira *et al*., 2015). Protein components from plant sources have also demonstrated anticancer effects in different cell lines (Enrique and Lizette, 2017).

Brewers' spent grain (BSG) is the insoluble part (mainly the seed coat–pericarp–husk) of barley grain remaining after the mashing step in the brewing process. With the large amounts produced annually, present low market value and being a nutritionally valuable co-product, BSG is being recognised as having potential use as a source of functional food components (Lynch *et al*., 2016). BSG protein hydrolysates have previously shown anti-inflammatory activity in Jurkat T cells (McCarthy *et al*., 2013a; McCarthy *et al*., 2013b). Additionally, phenolic extracts from BSG have demonstrated cellular antioxidant potential in U937 cells and HepG2 cells (Crowley *et al*., 2017 and Chapter 3). BSG is known as a source of hydroxycinnamic acids (McCarthy *et al*., 2013c), which are an important group of antioxidant compounds, known to have inhibitory activity in the proliferation of cancer cell lines (Roleira *et al*., 2015). The purpose of this research was to assess the antiproliferative potential of phenolic extracts and protein hydrolysates from BSG in cell lines from a cancerous origin. These cell lines used include RAW 264.7 cells - a murine macrophage cell line, Jurkat T cells - a human leukemic cell line, MCF-7 cells – a human breast cancer cell line, U937 cells - a human leukemic monocytic lymphoma cell line and HepG2 cells - a human liver carcinoma cell line. The MTT assay was used to assess cell proliferation. The

Hoechst staining and DNA fragmentation assays were used to measure apoptosis in U937 cells. All BSG phenolic extracts and protein hydrolysates studied in this thesis were included for analysis in this chapter.

Materials and methods

Materials

BSG was obtained from a single batch which was brewed in November 2009, vacuum-packed and stored at -20^{o}C in polypropylene bags until use. Shearzyme 500 L (declared activity 500 Fxu g^{-1}) and Ultraflo Max (declared activity 470 Fxu g^{-1}) were sourced from Novozymes A/S, Bagsværd, Denmark. Depol 740L (declared activity 945 u g^{-1}) was purchased from Biocatalysts Ltd., Cardiff, UK. Alcalase 2.4L and Flavourzyme® 500L were purchased from Sigma Chemical Co. (Dorset, UK), Brewer's Clarex™ (*Aspergillus niger* prolyl endoproteinase) was received from Dutch State Mines (DSM, Heerlen, Netherlands), Corolase® PP was supplied by AB Enzymes (Darmstadt, Germany) and Prolyve 1000 (Pro1k) was kindly provided by Lyven Enzymes Industrielles (Caen, France). Jurkat T cells, RAW 264.7 cells, MCF-7 cells, U937 cells and HepG2 cells were obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). All other chemicals were purchased from Sigma, unless otherwise stated.

Preparation of phenolic extracts

The preparation of BSG phenolic extracts was previously described by Crowley *et al*. (2017) and also detailed in Chapter 3 of thesis. Phenolic preparations from black, wet BSG were designated BW1-4; from black, dry BSG were designated BD1-2; from pale, wet BSG were designated PW1-5 and from pale, dry BSG were designated PD1-3 (Table 1, Chapter 3).

Generation of the alkaline-extracted protein hydrolysates

The alkaline extracted hydrolysates (44% w/w protein) were prepared as previously described (Connolly *et al*., 2013). The samples were then subjected to hydrolysis using combinations of Alcalase (Alc), Corolase PP (CorPP), Flavourzyme (Fla), Prolyve 1000 (Pro) and/or Protease P (Pro) producing the hydrolysates AlcFla, AlcPro, CorPPFla and ProPro. These alkaline-extracted BSG protein hydrolysates were labelled A1-A4, respectively (See Table 1, Chapter 6).

Simulated gastrointestinal digestion (SGID) of alkaline-extracted protein hydrolysates

SGID of BSG alkaline-extracted hydrolysates was performed according to Walsh *et al*. (2004). The samples were labelled S1-S4 (See Table 1, Chapter 6).

Generation of the enzyme-extracted protein hydrolysates (E1-E4)

The enzyme-extracted hydrolysates were labelled E1-E4 and are more fully described in Table 1, Chapter 6.

Membrane fractionation of alkaline-extracted protein hydrolysates

The hydrolysates were labelled U1-U6 and are more fully described in Table 2, Chapter 6.

Generation of the protein hydrolysates using combinations of Alcalase, Brewers Clarex and Flavourzyme (A-J)

The hydrolysates generated using combinations of Alcalase, Brewers Clarex and Flavourzyme were labelled A-J and are more fully described in Table 1, Chapter 5.

Cell culture

Jurkat T cells and U937 cells were maintained in antibiotic-free Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and plated at a density of 1 x 10^5 and 2 x 10^5 cells mL^{-1} for the cell proliferation assay, respectively. U937 cells were plated at a density of 1 x 10^5 and 2 x 10^6 cells mL^{-1} for the Hoechst staining and DNA fragmentation assay, respectively. RAW 264.7 cells, MCF-7 cells and HepG2 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) media supplemented with 10% FBS. Cells were seeded at a density of 0.2 x 10^5 and 0.5 x 10^5 cells mL^{-1} for the cell proliferation assay, respectively. Cells were maintained at 37°C in a 5% CO_2 atmosphere. Reduced serum media (2.5% FBS) was used for all experiments.

Cell Proliferation

The (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to measure the effect of the extracts on cell proliferation in all cell lines. Cells were incubated for 24 h at 37°C with BSG extracts at concentrations of 0 to 20% (v/v) (phenolic extracts) or 0 to 0.2% (w/v) (protein extracts). The MTT assay kit (MTT I proliferation kit, Roche Diagnostics, West Sussex, UK) was then used to measure cell proliferation. Absorbance was determined at 570 nm using a microplate reader (VarioskanFlash, Thermo Scientific, Waltham, MA) and cell proliferation was calculated as a percentage of the control, untreated cells. An inhibitory concentration-50 (IC_{50}) value, the concentration of a compound that reduces cell proliferation to 50% of the untreated control cells, was determined for each of the BSG samples.

Determination of apoptosis

The nuclear morphology of U937 cells treated with BSG phenolic extracts (IC_{50} values detailed in Crowley *et al*. (2017) and Table 3, Chapter 3) or Doxorubicin (0.5 µg mL^{-1}) was measured by fluorescence microscopy after staining with Hoechst 33342. After 24 hour incubation, approximately 2 x 10^5 cells mL^{-1} were

harvested by centrifugation (200 *g*, 10 minutes) to form a pellet. Hoechst 33342 stain (5 µg mL^{-1} PBS) was added and the samples were incubated at 37°C for 30 minutes. Stained samples were placed on a microscope slide and examined under UV light (330–380 nm) using a Nikon fluorescence microscope (200x magnification). A total of 300 cells in each sample were analysed and the percentage of condensed/fragmented (apoptotic) nuclei was calculated.

DNA fragmentation assay

Detection of small DNA fragments was carried out as previously described (O'Callaghan *et al*., 2010). Briefly, 2 x 10^5 cells mL^{-1} were harvested and the pellets were lysed; RNAse A (0.25 mg mL^{-1}) was added and the samples were incubated at 50°C for 1 h. Proteinase K (5 mg mL^{-1}) was added and the samples were incubated at 50°C for a further hour. Both the samples and a 100– 1500 bp DNA standard (Promega, Medical Supply Co. Ltd, Dublin, Ireland) were loaded to the wells of a 1.5% agarose gel and electrophoresis was performed in TBE buffer (0.45 M tris(hydroxymethyl) aminomethane, 0.45 M boric acid, and 2 mM EDTA, pH 8), at 3 V cm^{-1}. The gel was visualised using a UV transilluminator (312 nm) and photographed using an image analysis system.

Statistical analysis

All data represent the mean ± standard error (SE) of at least three independent experiments. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Dunnett's test using GraphPad Prism 4 (GraphPad software, La Jolla, CA).

Results

Antiproliferative effects of BSG phenolic extracts

The antiproliferative effects of BSG phenolic extracts were assessed in the Jurkat, RAW and MCF-7 cell lines. In Jurkat T cells, dose-dependent antiproliferative

effects were noted with the addition of BSG phenolic extracts (Table 1). Phenolic extracts from black, wet BSG (BW1-BW4), were more cytotoxic to this cancer cell line than other extracts, as noted by the lower IC_{50} values (1.4 – 3.1% v/v).

With the exception of the black BSG control (BW1), all black BSG extracts demonstrated statistically significant ($P<0.05$) toxic effects in RAW 264.7 cells, across the concentration range tested (Table 2). The extract, PW3, from pale, wet BSG also demonstrated cytotoxic effects at all concentrations. Extracts PW2 and PW3 were produced using the same volume of carbohydrase, 100 $\mu Lg^{-}BSG_{dw}$ Depol 740L. PW3 was produced using a pH 7, while PW2 was generated using pH5. However, extract PW2 did not demonstrate any cytotoxic effects, suggesting that the change in pH may have had a significant impact on the extract's toxicity profile. Apart from PW3, IC_{50} values could not be calculated for extracts from wet, pale BSG.

All black BSG phenolic extracts exhibited dose-dependent antiproliferative effects in MCF-7 cells (Table 3). The phenolic extracts had a lesser impact on MCF-7 cells compared to RAW cells, as noted by the inability to calculate IC_{50} values for all extracts apart from BW2, in MCF-7 cells.

Antiproliferative effects of BSG protein hydrolysates

The impact of BSG protein hydrolysates on the cell proliferation was assessed in the cell lines; Jurkat T cells, U937 cells, HepG2 cells and MCF-7 cells (Tables 4-14). Protein hydrolysates produced using either alkaline or enzyme hydrolysis (A1-E4) were assessed in Jurkat T cells at a concentration range between 0-0.2% (w/v) (Table 4). Dose-dependent decreases in cell proliferation were noted with alkaline-extracted hydrolysates (A1-A4) and enzyme-extracted hydrolysates (E1-E4). The impact of alkaline-extracted hydrolysates subjected to SGID (S1-S4), on Jurkat T cell proliferation, was far less, considering that IC_{50} values could not be calculated for these extracts. Alkaline-extracted hydrolysates subjected to membrane fractionation (U1-U6) did not have any significant impact on the proliferation of Jurkat T cells (Table 5).

Table 1: Effect of brewers' spent grain (BSG) phenolic extracts (0-20% (v/v)) on cell proliferation in Jurkat T cells as measured by (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

	0.5%	1%	2%	2.5%	4%	6%	8%	10%	15%	20%	IC_{50}
BW1	55.1 ± 4.1*	55.1 ± 13.5*	52.1 ± 10.1*	44.5 ± 14.8*	42.0 ± 21.2*	41.6 ± 7.9*	15.7 ± 9.7*	0.0 ± 0.0*	5.9 ± 5.9*	0.0 ± 0.0*	1.4
BW2	69.5 ± 6.5	64.9 ± 9.4	54.3 ± 27.3	63.1 ± 10.8	63.7 ± 3.0	48.7 ± 13.7*	31.8 ± 11.9*	28.9 ± 4.1*	0.0 ± 0.0*	1.3 ± 1.3*	3.1
BW3	66.1 ± 6.2	66.9 ± 13.8	57.4 ± 9.8	58.8 ± 22.2	48.6 ± 19.2	34.5 ± 19.3	33.1 ± 23.8	43.8 ± 17.8	19.3 ± 19.3*	7.7 ± 4.5*	2.9
BW4	57.4 ± 6.2	49.5 ± 2.0*	76.7 ± 7.9	39.4 ± 12.3*	60.6 ± 11.3	60.8 ± 21.7	16.7 ± 16.7*	6.2 ± 6.2*	0.0 ± 0.0*	15.7 ± 15.7*	2.1
BD1	56.5 ± 9.0	54.8 ± 1.7*	39.9 ± 8.4*	52.7 ± 12.4*	45.1 ± 19.3*	64.5 ± 9.2	46.1 ± 6.3*	57.1 ± 5.9	62.1 ± 18.2	37.2 ± 8.5*	n/d
BD2	61.0 ± 4.3	66.7 ± 2.2	58.7 ± 6.8	65.5 ± 19.9	51.6 ± 13.4*	64.0 ± 14.2	48.0 ± 15.3*	30.2 ± 11.3*	11.5 ± 7.7*	0.1 ± 0.1*	3.5
PW1	88.2 ± 2.5	79.5 ± 10.1	84.5 ± 7.6	87.3 ± 12.1	87.8 ± 9.6	86.3 ± 10.4	90.7 ± 4.2	84.7 ± 2.7	80.2 ± 5.7	90.7 ± 13.9	n/d
PW2	90.8 ± 11.9	89.2 ± 14.6	91.4 ± 20.4	87.3 ± 10.5	81.4 ± 6.6	80.5 ± 12.0	88.4 ± 9.7	81.0 ± 5.3	68.1 ± 10.3	48.9 ± 7.6*	n/d
PW3	111.0 ± 2.5	112.8 ± 1.3	114.1 ± 5.4	114.2 ± 6.8	100.1 ± 1.2	95.0 ± 4.0	74.2 ± 7.9*	80.4 ± 1.7*	77.2 ± 1.6*	69.1 ± 0.3*	29.7
PW4	87.9 ± 5.3	89.4 ± 6.6	79.0 ± 5.4	108.0 ± 22.3	94.2 ± 12.7	74.9 ± 7.3	88.6 ± 18.5	84.9 ± 13.5	49.0 ± 12.9*	49.0 ± 4.1*	n/d
PW5	99.5 ± 14.8	63.1 ± 3.9	86.0 ± 2.8	98.9 ± 15.2	79.9 ± 5.3	76.2 ± 17.6	66.2 ± 10.2	70.8 ± 7.0	44.6 ± 12.0*	49.5 ± 15.6*	19.5
PD1	108.1 ± 13.6	120.2 ± 14.5	105.7 ± 7.4	107.5 ± 10.0	103.9 ± 8.3	94.6 ± 9.3	80.8 ± 10.4	84.2 ± 16.8	93.6 ± 11.6	64.8 ± 10.1	n/d
PD2	113.8 ± 10.0	108.0 ± 11.5	94.2 ± 2.2	90.5 ± 2.9	88.9 ± 2.0	94.8 ± 4.6	73.0 ± 8.3	67.7 ± 7.6*	72.8 ± 5.6	51.4 ± 12.6*	22.7
PD3	108.4 ± 5.1	124.2 ± 7.2*	113.1 ± 6.0	117.2 ± 6.1	105.5 ± 4.7	104.5 ± 2.5	100.7 ± 5.7	96.9 ± 3.6	85.6 ± 3.2	79.3 ± 7.5	29.6

Values are mean ± SE of three independent experiments, expressed as a percentage relative to untreated Jurkat T cells. BW1-4 represent extracts from black, wet BSG; BD1-2 represents extracts from black, dry BSG; PW1-5 represents extracts from pale, wet BSG and PD1-3 represents extracts from pale, dry BSG. Statistical analysis by ANOVA followed by Dunnett's Test. * Denotes statistically significant difference in cell proliferation, relative to untreated Jurkat T cells ($P<0.05$). n/d: IC_{50} was not determinable for the range of concentrations tested.

Table 2: Effect of brewers' spent grain (BSG) phenolic extracts (0-20% (v/v)) on cell proliferation in RAW 264.7 cells as measured by (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

	0.5%	1%	2%	2.5%	4%	6%	8%	10%	15%	20%	IC_{50}
BW1	50.0 ± 5.2	71.1 ± 23.6	45.6 ± 11.1	47.2 ± 7.3	60.5 ± 17.0	65.2 ± 19.9	67.1 ± 19.1	51.1 ± 9.7	61.6 ± 20.2	59.7 ± 11.3	0.05
BW2	40.9 ± 9.2*	46.2 ± 2.0*	46.4 ± 3.4*	41.4 ± 2.6*	50.7 ± 1.5*	47.5 ± 5.3*	54.4 ± 3.2*	46.0 ± 0.4*	59.5 ± 6.5*	49.7 ± 6.6*	7.0
BW3	54.2 ± 4.6*	54.3 ± 8.9*	49.1 ± 7.3*	49.2 ± 6.5*	50.2 ± 6.8*	47.1 ± 4.0*	49.8 ± 8.7*	56.6 ± 9.0*	53.8 ± 3.2*	54.0 ± 2.2*	n/d
BW4	44.1 ± 6.8*	55.2 ± 7.2*	46.2 ± 1.9*	42.9 ± 4.7*	53.8 ± 5.1*	52.6 ± 3.6*	53.8 ± 1.2*	51.9 ± 5.6*	50.3 ± 4.6*	56.1 ± 6.0*	3.0
BD1	58.1 ± 8.0*	48.0 ± 7.4*	45.0 ± 9.7*	43.9 ± 5.1*	42.8 ± 7.2*	50.8 ± 10.3*	54.0 ± 8.7*	44.7 ± 2.9*	54.9 ± 9.5*	61.2 ± 16.1*	3.1
BD2	51.6 ± 5.8*	48.5 ± 9.8*	45.3 ± 4.2*	44.8 ± 2.8*	44.7 ± 3.9*	49.4 ± 7.8*	53.1 ± 7.0*	65.9 ± 15.3*	51.1 ± 5.1*	55.6 ± 5.2*	2.8
PW1	105.0 ± 2.2	109.3 ± 1.2	109.1 ± 6.5	118.9 ± 18.4	124.0 ± 16.9	112.1 ± 11.3	123.2 ± 8.4	104.7 ± 4.3	122.2 ± 19.7	129.1 ± 25.7	n/d
PW2	140.5 ± 9.5	141.1 ± 20.2	135.4 ± 1.2	136.6 ± 6.2	138.1 ± 14.2	136.9 ± 3.2	138.9 ± 11.0	124.4 ± 5.1	132.6 ± 23.9	141.0 ± 19.5	n/d
PW3	72.7 ± 12.2*	70.4 ± 7.5*	53.9 ± 6.6*	51.2 ± 2.9*	42.2 ± 2.2*	46.5 ± 5.8*	50.1 ± 5.9*	48.7 ± 5.1*	45.1 ± 4.3*	47.6 ± 5.6*	6.1
PW4	95.8 ± 19.5	107.6 ± 6.8	84.7 ± 8.3	83.0 ± 4.5	76.3 ± 9.7	100.6 ± 8.8	74.7 ± 4.9	85.7 ± 12.6	74.8 ± 7.9	60.9 ± 10.2	n/d
PW5	100.7 ± 9.0	88.9 ± 17.8	70.5 ± 10.9	68.7 ± 12.0	68.5 ± 11.4	73.7 ± 11.2	68.0 ± 9.3	63.9 ± 11.7	62.7 ± 6.7	70.4 ± 14.4	n/d
PD1	99.8 ± 22.4	98.7 ± 29.7	101.1 ± 28.2	104.7 ± 30.4	92.2 ± 19.6	65.0 ± 2.6	64.7 ± 13.9	45.8 ± 6.8	60.9 ± 18.2	46.3 ± 6.8	14.8
PD2	88.1 ± 8.4	88.4 ± 10.2	81.8 ± 5.2	78.8 ± 11.3	74.4 ± 11.7	56.6 ± 7.7*	56.5 ± 7.6*	53.9 ± 8.3*	47.5 ± 6.0*	39.9 ± 7.1*	11.9
PD3	91.4 ± 10.1	87.0 ± 6.9	89.8 ± 4.4	85.4 ± 5.7	78.1 ± 13.1	71.9 ± 9.0	61.0 ± 3.1*	57.2 ± 2.5*	60.2 ± 10.9*	47.5 ± 1.6*	18.6

Values are mean ± SE of three independent experiments, expressed as a percentage relative to untreated RAW 264.7 cells. BW1-4 represent extracts from black, wet BSG; BD1-2 represents extracts from black, dry BSG; PW1-5 represents extracts from pale, wet BSG and PD1-3 represents extracts from pale, dry BSG. Statistical analysis by ANOVA followed by Dunnett's Test. * Denotes statistically significant difference in cell proliferation, relative to untreated RAW 264.7 cells ($P<0.05$). n/d: IC_{50} was not determinable for the range of concentrations tested.

Table 3: Effect of brewers' spent grain (BSG) phenolic extracts (0-20% (v/v)) on cell proliferation in MCF-7 cells as measured by (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

	0.5%	1%	2%	2.5%	4%	6%	8%	10%	15%	20%	IC_{50}
BW1	82.5 ± 3.7	88.0 ± 12.0	64.2 ± 5.0*	67.3 ± 3.8*	68.1 ± 6.8*	66.1 ± 6.2*	65.6 ± 4.4*	72.4 ± 7.2	71.8 ± 10.0	66.7 ± 12.2*	n/d
BW2	74.2 ± 9.3	67.9 ± 5.5*	60.8 ± 4.4*	64.0 ± 5.3*	57.6 ± 4.1*	58.5 ± 4.6*	60.7 ± 2.1*	39.4 ± 8.5*	49.4 ± 10.5*	41.9 ± 15.7*	10.9
BW3	79.3 ± 2.6	66.4 ± 7.3*	68.8 ± 8.6	63.3 ± 7.4*	65.7 ± 8.3*	65.1 ± 3.2*	51.6 ± 9.5*	58.2 ± 10.6*	59.0 ± 5.9*	48.6 ± 11.1*	n/d
BW4	68.7 ± 7.6	63.8 ± 11.1	60.7 ± 5.5*	60.8 ± 8.0*	57.4 ± 7.7*	58.6 ± 8.6*	57.5 ± 12.9*	56.7 ± 7.8*	55.7 ± 9.3*	49.8 ± 13.7*	n/d
BD1	83.9 ± 8.4	74.2 ± 6.0	68.2 ± 12.3	66.3 ± 4.3	70.9 ± 12.1	74.9 ± 7.3	71.8 ± 13.8	65.7 ± 6.9	66.6 ± 10.5	56.5 ± 5.6*	n/d
BD2	68.7 ± 6.0	69.4 ± 7.2	58.6 ± 5.8*	62.4 ± 5.1*	56.0 ± 5.1*	60.5 ± 4.0*	63.3 ± 10.1*	57.3 ± 9.1*	52.7 ± 18.3*	53.9 ± 9.5*	n/d
PW1	106.4 ± 3.5	94.6 ± 1.7	93.6 ± 4.9	92.8 ± 3.1	87.9 ± 13.9	87.0 ± 3.5	91.5 ± 4.5	94.9 ± 3.0	85.5 ± 1.3	70.7 ± 12.9*	n/d
PW2	94.4 ± 9.7	94.5 ± 11.7	89.5 ± 11.5	87.5 ± 13.9	108.5 ± 14.5	103.1 ± 11.2	90.1 ± 4.0	94.8 ± 8.8	92.0 ± 3.5	72.6 ± 18.4	n/d
PW3	95.0 ± 6.1	94.0 ± 7.5	95.5 ± 6.0	88.1 ± 10.6	85.7 ± 16.1	93.0 ± 0.5	93.2 ± 2.0	92.0 ± 4.0	100.0 ± 3.8	95.6 ± 5.4	n/d
PW4	88.2 ± 8.4	93.8 ± 12.7	87.9 ± 11.2	88.0 ± 9.8	83.6 ± 7.4	79.0 ± 18.4	98.1 ± 23.7	87.2 ± 12.8	101.1 ± 16.4	67.9 ± 15.8	n/d
PW5	95.2 ± 10.2	83.9 ± 6.9	82.1 ± 4.7	84.1 ± 6.7	91.8 ± 10.5	90.3 ± 11.0	80.2 ± 7.6	84.8 ± 15.0	87.3 ± 7.2	62.1 ± 11.6*	n/d
PD1	90.3 ± 6.0	86.5 ± 4.2	79.5 ± 4.6	75.5 ± 4.9	69.1 ± 2.7*	74.7 ± 5.9	76.4 ± 10.1	72.8 ± 4.7*	66.8 ± 12.3*	72.4 ± 9.6*	n/d
PD2	99.4 ± 3.7	96.5 ± 5.1	83.8 ± 2.5	83.5 ± 2.9	75.3 ± 5.1*	79.6 ± 3.6	82.6 ± 7.3	70.5 ± 8.8*	82.3 ± 5.1	81.0 ± 7.4	n/d
PD3	90.6 ± 3.2	90.7 ± 3.8	81.0 ± 3.0	78.4 ± 6.5	83.5 ± 10.7	91.5 ± 9.6	87.8 ± 4.9	85.3 ± 3.4	96.7 ± 11.1	72.0 ± 19.3	n/d

Values are mean ± SE of three independent experiments, expressed as a percentage relative to untreated MCF-7 cells. BW1-4 represent extracts from black, wet BSG; BD1-2 represents extracts from black, dry BSG; PW1-5 represents extracts from pale, wet BSG and PD1-3 represents extracts from pale, dry BSG. Statistical analysis by ANOVA followed by Dunnett's Test. * Denotes statistically significant difference in cell proliferation, relative to untreated MCF-7 cells ($P<0.05$). n/d: IC_{50} was not determinable for the range of concentrations tested.

Table 4: Effects of brewers' spent grain (BSG) protein hydrolysates A1-E4 (0-0.2% (w/v)) on cell proliferation in the Jurkat T cells as measured by (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

	0.005%	0.01%	0.02%	0.025%	0.04%	0.06%	0.08%	0.1%	0.15%	0.2%	IC_{50}
A1	100.8±9.6	95.9±4.5	85.6±1.9	99.4±5.2	81.5±21.8	45.3±20.0*	18.6±0.4*	21.5±3.9*	25.1±4.3*	21.4±11.2*	0.06
S1	64.7±11.2	58.9±13.1*	62.0±13.2*	59.4±7.8*	71.9±6.0	60.7±10.2*	69.5±6.1	72.6±10.4	65.3±6.8	41.4±8.2*	n/d
E1	68.2±16.2	60.1±16.0	50.2±17.1	47.0±12.5*	52.8±12.7	53.0±8.7	46.6±13.6	52.1±12.7	30.6±9.8*	29.8±12.7*	0.04
A2	102.6±5.8	99.9±13.0	90.8±16.1	86.0±14.4	109.9±14.1	58.9±22.9	29.7±15.2*	13.3±2.5*	19.1±3.1*	16.4±5.0*	0.07
S2	62.1±18.7	67.9±19.7	77.3±24.3	75.0±16.8	80.2±16.4	81.7±16.5	85.9±16.3	67.3±19.1	91.5±22.7	75.7±17.6	n/d
E2	78.0±15.4	69.3±12.0	58.0±14.5	62.5±11.4	70.7±20.7	63.1±4.9	62.3±4.9	61.4±8.4	71.1±12.1	63.6±6.5	n/d
A3	93.1±7.2	95.3±4.8	91.1±1.3	94.9±4.1	105.7±3.5	72.0±13.8	53.4±24.2*	9.4±2.4*	27.2±5.7*	11.4±5.1*	0.08
S3	69.0±12.4	63.1±11.3	64.6±10.5	65.7±13.8	66.6±12.2	62.3±10.7	69.0±8.7	65.5±11.3	62.3±13.5	59.2±5.8	n/d
E3	77.0±15.3	71.5±17.8	71.5±18.6	63.3±15.0	54.6±12.3	49.5±10.5	64.1±13.3	59.6±15.3	41.5±13.3*	35.5±10.6*	0.1
A4	95.1±19.6	108.0±14.7	110.3±9.0	103.5±11.9	116.3±14.4	66.7±14.6	42.1±3.2*	25.7±9.8*	33.0±8.6*	16.2±0.3*	0.08
S4	75.7±14.5	64.8±15.7	69.1±13.7	62.6±15.2	68.1±11.2	62.4±10.4	70.6±11.7	70.9±8.9	65.2±19.0	58.4±8.2	n/d
E4	58.2±9.9	50.6±12.1*	53.3±11.3*	54.9±13.0*	50.9±13.9*	51.5±11.7*	48.6±12.8*	49.5±12.0*	47.5±10.1*	39.5±6.0*	0.05

Values are mean ± SE of at least three independent experiments. Statistical analysis was by ANOVA followed by Dunnett's test. * Denotes significant difference ($P<0.05$) in cell proliferation relative to untreated control Jurkat T cells. n/d: IC_{50} was not determinable for the range of concentrations tested. A1 is a BSG protein hydrolysate prepared via alkaline hydrolysis and subjected to further hydrolysis by ProPro. S1 is A1 subjected to SGID. E1 is prepared via direct enzyme hydrolysis by ProPro. A2 is a BSG protein hydrolysate prepared via alkaline hydrolysis and subjected to further hydrolysis by CorPPFla. S2 is A2 subjected to SGID. E2 is prepared via direct enzyme hydrolysis by CorPPFla. A3 is a BSG protein hydrolysate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcPro. S3 is A3 subjected to SGID. E3 is prepared via direct enzyme hydrolysis by AlcPro. A4 is a BSG protein hydrolysate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcFla. S4 is A4 subjected to SGID. E4 is prepared via direct enzyme hydrolysis by AlcFla.

Table 5: Effects of brewers' spent grain (BSG) protein hydrolysates U1-U6 (0-0.2% (w/v)) on cell proliferation in the Jurkat T cells as measured by (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

	0.005	0.01	0.02	0.025	0.04	0.06	0.08	0.1	0.15	0.2	IC_{50}
U1	85.5±3.2	99.6±5.3	87.7±13.5	95.6±11.8	92.8±8.6	91.6±5.9	90.9±20.8	79.7±16.6	59.7±13.4	75.4±9.0	0.5
U2	81.5±7.8	93.0±11.6	94.9±9.4	97.4±9.3	105.4±8.7	95.3±8.1	88.3±14.7	109.8±13.0	104.9±9.4	99.3±18.9	n/d
U3	86.3±8.4	92.7±11.3	93.5±7.4	84.4±16.6	96.0±12.1	86.9±3.4	87.2±10.5	86.1±9.3	67.3±3.9	81.7±15.3	n/d
U4	92.9±13.1	106.9±14.5	112.5±15.1	93.2±21.1	95.1±10.1	106.6±12.5	102.0±7.4	91.5±17.5	84.5±11.5	87.6±3.2	0.5
U5	94.9±23.4	108.8±14.5	104.1±19.9	110.8±2.7	95.6±1.4	99.8±19.9	77.5±17.1	89.6±5.7	95.6±2.0	82.5±14.7	0.7
U6	72.8±9.9	89.1±15.1	99.7±21.3	104.7±18.2	100.6±24.5	93.0±9.5	94.9±22.5	100.5±23.7	84.6±21.2	81.7±15.3	n/d

Values are mean ± SE of at least three independent experiments. Statistical analysis was by ANOVA followed by Dunnett's test. n/d: IC_{50} was not determinable for the range of concentrations tested. U1 is a 30kDa Retentate prepared via alkaline hydrolysis and subjected to further hydrolysis by CorPPFla. U2 is a 30kDa Retentate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcFla. U3 is a 30kDa Permeate prepared via alkaline hydrolysis and subjected to further hydrolysis by CorPPFla. U4 is a 30kDa Permeate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcFla. U5 is a 10kDa Permeate prepared via alkaline hydrolysis and subjected to further hydrolysis by CorPPFla. U6 is a 10kDa Permeate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcFla.

Table 6: Effects of brewers' spent grain (BSG) protein hydrolysates A1-E4 (0-0.2% (w/v)) on cell proliferation in the U937 cells as measured by (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

	0.005	0.01	0.02	0.025	0.04	0.06	0.08	0.1	0.15	0.2	IC_{50}
A1	81.3 ± 8.1	84.6 ± 11.9	84.9 ± 9.3	95.4 ± 9.4	87.8 ± 4.8	72.6 ± 12.4	63.1 ± 10.1	33.8 ± 11.9*	29.9 ± 7.2*	36.9 ± 9.5*	0.1
S1	71.6 ± 9.7*	71.0 ± 4.7*	63.8 ± 4.4*	66.5 ± 6.5*	65.5 ± 3.0*	63.3 ± 4.8 *	57.9 ± 12.2*	71.9 ± 4.6*	71.5 ± 6.8*	70.4 ± 5.3*	n/d
E1	86.2 ± 4.1	86.6 ± 8.2	89.1± 13.3	78.0 ± 9.5	70.9 ± 7.9	69.8 ± 8.2*	58.7 ± 8.8*	70.9 ± 7.6	47.8 ± 3.4*	42.9 ± 4.7*	0.18
A2	77.8 ± 6.3	76.3 ± 3.1	86.2 ±6.3	93.1 ± 7.3	103.1 ± 10.3	90.5 ± 12.3	76.9 ± 21.3	34.2 ± 14.1*	17.2 ± 4.1*	17.4 ± 2.6*	0.1
S2	86.9 ± 9.8	88.7 ± 14.2	89.1 ± 7.4	86.3 ± 13.6	91.4 ± 9.7	91.1 ± 10.5	96.0 ± 9.9	90.4 ± 10.0	95.1 ± 15.2	77.3 ± 9.4	n/d
E2	82.2 ± 4.4	83.2 ± 8.6	80.9 ± 2.9	81.4 ± 3.5	79.4 ± 2.9	86.9 ± 6.0	67.6 ± 7.4*	74.3 ± 3.0	91.2 ± 14.6	94.4 ± 15.5	n/d
A3	72.0 ± 6.1	75.2 ± 3.5	89.3 ± 7.6	87.0 ± 1.9	101.1 ± 5.3	81.0 ± 13.7	89.6 ± 14.6	73.7 ± 18.8	14.0 ± 1.7*	14.0 ± 2.4*	0.12
S3	101.3 ± 4.4	89.8 ± 2.6	99.1 ± 9.4	80.2 ± 7.0	89.6 ± 5.4	83.1 ± 4.4	88.7 ± 3.6	85.9 ± 5.8	91.0 ± 10.9	84.1 ± 10.3	n/d
E3	78.1 ± 6.4	77.9 ± 8.9	76.5 ± 3.0	74.7 ± 5.7	68.5 ± 4.9*	61.6 ± 15.3*	64.2 ± 5.5*	64.7 ± 3.6*	62.5 ± 7.6*	60.9 ± 8.8*	0.87
A4	76.6 ±10.2	91.8 ± 7.5	88.2 ± 6.5	100.1 ± 6.6	109.4 ± 10.5	90.3 ± 25.7	82.5 ± 20.8	71.0 ± 17.7	24.2 ± 8.2*	27.4 ± 9.3*	0.12
S4	87.8 ± 5.3	82.7 ± 6.3	85.8 ± 5.2	73.8 ± 6.1	72.8 ± 3.8	62.7 ± 18.5*	89.5 ± 10.2	76.8 ± 5.8	81.2 ± 7.2	81.0 ± 4.3	n/d
E4	66.7 ± 3.4*	70.7 ± 8.6*	59.9 ± 4.8*	60.0 ± 2.5*	57.6 ± 9.4*	55.5 ± 2.8*	64.5 ± 7.8*	63.5 ± 6.0*	59.9 ± 4.7*	61.6 ± 5.2*	n/d

Values are mean ± SE of at least three independent experiments. Statistical analysis was by ANOVA followed by Dunnett's test. * Denotes significant difference ($P<0.05$) in cell proliferation relative to untreated control U937 cells. n/d: IC_{50} was not determinable for the range of concentrations tested. A1 is a BSG protein hydrolysate prepared via alkaline hydrolysis and subjected to further hydrolysis by ProPro. S1 is A1 subjected to SGID. E1 is prepared via direct enzyme hydrolysis by ProPro. A2 is a BSG protein hydrolysate prepared via alkaline hydrolysis and subjected to further hydrolysis by CorPPFla. S2 is A2 subjected to SGID. E2 is prepared via direct enzyme hydrolysis by CorPPFla. A3 is a BSG protein hydrolysate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcPro. S3 is A3 subjected to SGID. E3 is prepared via direct enzyme hydrolysis by AlcPro. A4 is a BSG protein hydrolysate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcFla. S4 is A4 subjected to SGID. E4 is prepared via direct enzyme hydrolysis by AlcFla.

Table 7: Effects of brewers' spent grain (BSG) protein hydrolysates U1-U6 (0-0.2% (w/v)) on cell proliferation in the U937 cells as measured by (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

	0.005	0.01	0.02	0.025	0.04	0.06	0.08	0.1	0.15	0.2	IC_{50}
U1	91.5 ± 7.8	97.0 ± 5.1	93.8 ± 4.9	99.3 ± 1.6	88.1 ± 10.1	98.1 ± 3.7	84.8 ± 2.3	68.9 ± 13.8	48.7 ± 24.6*	71.0 ± 4.1	0.26
U2	69.6 ± 14.1	76.9 ± 13.1	74.9 ± 9.3	81.3 ± 13.6	66.6 ± 7.1	89.6 ± 11.2	87.6 ± 10.3	69.8 ± 5.0	70.6 ± 16.6	24.6 ± 23.6*	0.54
U3	102.6 ± 15.8	97.7 ± 18.2	86.7 ± 17.6	83.0 ± 10.4	86.8 ± 10.2	78.5 ± 7.7	68.7 ± 11.5	78.0 ± 13.2	87.1 ± 15.4	91.2 ± 20.2	n/d
U4	100.2 ± 11.5	120.0 ± 3.7	121.9 ± 6.7	102.7 ± 6.9	100.8 ± 3.4	113.4 ± 5.6	124.0 ± 16.1	104.5 ± 9.2	113.6 ± 11.5	98.3 ± 5.0	n/d
U5	90.2 ± 7.4	108.1 ± 14.7	94.1 ± 13.7	111.3 ± 14.7	101.0 ± 12.0	96.7 ± 23.4	104.7 ± 13.1	94.5 ± 10.8	99.5 ± 16.7	92.3 ± 15.8	n/d
U6	112.4 ± 6.2	131.9 ± 16.8	130.7 ± 14.5	139.9 ± 25.0	122.9 ± 9.3	122.7 ± 3.8	112.8 ± 5.9	111.7 ± 6.5	114.4 ± 7.1	109.2 ± 10.0	n/d

Values are mean ± SE of at least three independent experiments. Statistical analysis was by ANOVA followed by Dunnett's test. * Denotes significant difference ($P<0.05$) in cell proliferation relative to untreated control U937 cells. n/d: IC_{50} was not determinable for the range of concentrations tested. U1 is a 30kDa Retentate prepared via alkaline hydrolysis and subjected to further hydrolysis by CorPPFla. U2 is a 30kDa Retentate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcFla. U3 is a 30kDa Permeate prepared via alkaline hydrolysis and subjected to further hydrolysis by CorPPFla. U4 is a 30kDa Permeate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcFla. U5 is a 10kDa Permeate prepared via alkaline hydrolysis and subjected to further hydrolysis by CorPPFla. U6 is a 10kDa Permeate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcFla.

Table 8: Effects of brewers' spent grain (BSG) protein hydrolysates A-J (0-0.2% (w/v)) on cell proliferation in the U937 cells as measured by (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

	0.005	0.01	0.02	0.025	0.04	0.06	0.08	0.1	0.15	0.2	IC_{50}
A	84.9 ± 6.1	76.9 ± 0.8	80.5 ± 3.2	75.7 ± 5.9	72.9 ± 10.7	73.2 ± 9.7	75.9 ± 10.7	77.2 ± 12.7	76.1 ± 13.4	72.9 ± 9.0	n/d
B	78.8 ± 1.2	82.8 ± 5.6	75.2 ± 3.0	89.1 ± 17.7	102.0 ± 11.8	96.8 ± 22.4	90.4 ± 16.4	103.8 ± 26.4	109.2 ± 34.6	106.5 ± 40.8	n/d
C	78.3 ± 11.3	81.6 ± 17.4	90.7 ± 17.1	81.7 ± 6.8	77.3 ± 2.5	60.8 ± 28.7	81.3 ± 10.6	87.6 ± 4.8	89.0 ± 7.0	73.0 ± 11.3	n/d
D	84.6 ± 8.0	88.8 ± 10.4	92.9 ± 19.2	93.0 ± 18.7	93.0 ± 11.6	100.5 ± 11.6	90.6 ± 16.0	89.8 ± 15.9	65.1 ± 32.4	96.9 ± 10.0	n/d
E	61.6 ± 5.5*	75.5 ± 8.4	86.8 ± 8.2	82.4 ± 10.7	76.4 ± 11.1	82.0 ± 8.7	87.0 ± 9.1	89.2 ± 12.1	95.1 ± 8.7	89.6 ± 7.5	n/d
F	60.3 ± 3.5	63.5 ± 9.6	70.3 ± 12.3	78.0 ± 12.8	61.9 ± 13.9	80.1 ± 9.9	70.1 ± 13.1	77.7 ± 12.2	75.7 ± 13.4	74.9 ± 10.9	n/d
G	67.1 ± 9.6	66.4 ± 9.6	68.5 ± 10.0	70.8 ± 9.7	76.8 ± 16.8	67.7 ± 11.3	65.4 ± 7.7	69.9 ± 7.4	76.0 ± 10.4	69.3 ± 6.7	n/d
H	71.1 ± 15.0	75.0 ± 13.8	73.0 ± 15.2	76.6 ± 19.2	83.8 ± 14.2	77.3 ± 14.2	86.6 ± 11.1	88.5 ± 10.9	91.2 ± 20.4	94.7 ± 17.8	n/d
I	72.1 ± 6.3	76.3 ± 5.0	83.9 ± 10.8	77.4 ± 11.0	74.8 ± 10.8	81.8 ± 7.7	71.1 ± 9.5	75.8 ± 4.0	58.7 ± 20.0	78.5 ± 12.5	n/d
J	76.8 ± 8.7	82.9 ± 13.0	78.2 ± 11.2	88.0 ± 16.9	81.3 ± 18.4	85.3 ± 12.6	91.5 ± 9.8	85.0 ± 13.4	81.3 ± 15.1	82.0 ± 17.0	n/d

Values are mean ± SE of at least three independent experiments. Statistical analysis was by ANOVA followed by Dunnett's test. * Denotes significant difference ($P<0.05$) in cell proliferation relative to untreated control U937 cells. n/d: IC_{50} was not determinable for the range of concentrations tested. A is unhydrolysed BSG protein. B is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Brewers Clarex and Flavourzyme. C is BSG protein treated with Depol 740L, Brewers Clarex and Flavourzyme. D is BSG protein treated with Shearzyme, Ultraflo Max, Brewers Clarex and Flavourzyme. E is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Alcalase and Flavourzyme. F is BSG protein treated with Depol 740L, Alcalase and Flavourzyme. G is BSG protein treated with Shearzyme, Ultraflo Max, Alcalase and Flavourzyme. H is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. I is BSG protein treated with Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. J is BSG protein treated with Shearzyme, Ultraflo Max, Alcalase, Brewers Clarex and Flavourzyme.

Table 9: Effects of brewers' spent grain (BSG) protein hydrolysates A1-E4 (0-0.2% (w/v)) on cell proliferation in the HepG2 cells as measured by (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

	0.005	0.01	0.02	0.025	0.04	0.06	0.08	0.1	0.15	0.2	IC_{50}
A1	65.4 ± 6.8*	71.7 ± 4.4*	82.1 ± 3.8	76.3 ± 2.1*	78.8 ± 3.7*	83.3 ± 3.4	90.5 ± 3.4	80.1 ± 7.3	67.3 ± 7.0*	53.9 ± 5.7*	n/d
S1	87.2 ± 5.6	101.0 ± 21.5	103.6 ± 29.9	94.6 ± 19.1	93.4 ± 15.6	101.2 ± 19.8	91.6 ± 22.0	91.8 ± 20.6	92.6 ± 21.5	91.2 ± 15.0	n/d
E1	73.1 ± 8.5	69.5 ± 11.0	55.6 ± 11.0*	49.2 ± 8.1*	56.0 ± 5.9*	60.0 ± 6.9*	59.2 ± 9.2*	62.5 ± 5.7*	67.5 ± 3.9*	58.8 ± 6.6*	n/d
A2	57.7 ± 5.5*	50.4 ± 7.6*	57.5 ± 7.9*	52.1 ± 4.7*	51.8 ± 8.2*	76.3 ± 15.1	71.9 ± 4.2	62.1 ± 7.9*	60.6 ± 12.3*	38.9 ± 4.4*	n/d
S2	104.7 ± 11.8	84.6 ± 7.1	80.0 ± 12.8	66.5 ± 7.7	80.5 ± 7.4	88.0 ± 4.1	85.0 ± 2.7	85.2 ± 7.7	100.7 ± 8.6	82.6 ± 12.8	n/d
E2	70.0 ± 7.3*	73.7 ± 3.5*	59.3 ± 7.3*	56.0 ± 6.2*	58.6 ± 4.3*	62.6 ± 4.6*	66.0 ± 3.6*	68.5 ± 2.7*	76.1 ± 3.2*	62.6 ± 5.9*	n/d
A3	65.1 ± 7.5*	58.2 ± 8.7*	50.4 ± 9.4*	51.8 ± 12.0*	48.3 ± 10.3*	66.0 ± 10.6*	58.6 ± 5.7*	61.2 ± 4.0*	58.4 ± 7.9*	48.6 ± 3.2*	n/d
S3	107.4 ± 8.4	97.8 ± 15.8	84.0 ± 33.5	78.9 ± 12.7	95.3 ± 27.5	63.4 ± 4.8	95.8 ± 22.0	92.3 ± 20.9	102.5 ± 28.5	93.4 ± 14.8	n/d
E3	92.0 ± 9.7	86.8 ± 6.0	71.4 ± 4.2	67.8 ± 8.6	60.3 ± 4.7*	73.4 ± 9.6	81.8 ± 8.1	85.6 ± 12.2	77.5 ± 8.8	74.1 ± 8.1	n/d
A4	64.6 ± 5.8*	54.4 ± 7.6*	52.5 ± 6.3*	49.1 ± 9.8*	50.6 ± 8.5*	65.3 ± 5.9*	56.3 ± 6.2*	63.0 ± 5.1*	61.0 ± 6.2*	47.2 ± 6.6*	n/d
S4	103.2 ± 4.6	105.3 ± 10.7	87.8 ± 11.3	81.6 ± 8.6	83.5 ± 3.2	90.6 ± 9.1	80.5 ± 3.9	93.4 ± 8.3	97.5 ± 11.4	90.6 ± 11.7	n/d
E4	82.6 ± 3.2	84.9 ± 4.3	78.5 ± 4.0	74.9 ± 9.6	72.0 ± 7.0	74.9 ± 9.6	70.2 ± 5.6	88.3 ± 12.9	83.3 ± 8.3	69.1 ± 6.7*	n/d

Values are mean ± SE of at least three independent experiments. Statistical analysis was by ANOVA followed by Dunnett's test. * Denotes significant difference ($P<0.05$) in cell proliferation relative to untreated control HepG2 cells. n/d: IC_{50} was not determinable for the range of concentrations tested. A1 is a BSG protein hydrolysate prepared via alkaline hydrolysis and subjected to further hydrolysis by ProPro. S1 is A1 subjected to SGID. E1 is prepared via direct enzyme hydrolysis by ProPro. A2 is a BSG protein hydrolysate prepared via alkaline hydrolysis and subjected to further hydrolysis by CorPPFla. S2 is A2 subjected to SGID. E2 is prepared via direct enzyme hydrolysis by CorPPFla. A3 is a BSG protein hydrolysate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcPro. S3 is A3 subjected to SGID. E3 is prepared via direct enzyme hydrolysis by AlcPro. A4 is a BSG protein hydrolysate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcFla. S4 is A4 subjected to SGID. E4 is prepared via direct enzyme hydrolysis by AlcFla.

Table 10: Effects of brewers' spent grain (BSG) protein hydrolysates U1-U6 (0-0.2% (w/v)) on cell proliferation in the HepG2 cells as measured by (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

	0.005	0.01	0.02	0.025	0.04	0.06	0.08	0.1	0.15	0.2	IC_{50}
U1	69.7 ± 6.3*	64.4 ± 2.9*	62.8 ± 3.5*	60.0 ± 3.4*	52.6 ± 1.7*	76.3 ± 8.4	67.3 ± 2.6*	78.9 ± 15.3	76.7 ± 10.4	68.6 ± 7.9*	n/d
U2	84.9 ± 6.8	83.9 ± 13.2	79.2 ± 16.8	81.1 ± 17.9	74.5 ± 20.4	85.7 ± 21.7	85.3 ± 16.6	65.6 ± 5.1	60.3 ± 8.5	57.7 ± 7.6	0.7
U3	109.2 ± 3.0	117.5 ± 23.3	88.5 ± 12.4	87.5 ± 9.6	97.9 ± 12.8	95.5 ± 7.2	95.4 ± 6.9	95.7 ± 12.0	88.0 ± 13.4	106.4 ± 7.7	n/d
U4	79.9 ± 8.9	77.8 ± 16.3	79.8 ± 16.4	76.2 ± 20.2	77.8 ± 19.4	102.1 ± 14.0	85.1 ± 16.7	67.3 ± 3.8	68.0 ± 1.7	59.5 ± 9.4	n/d
U5	72.6 ± 7.0	69.3 ± 2.1	64.8 ± 3.2	60.9 ± 1.8	58.9 ± 6.6	82.9 ± 12.4	95.2 ± 25.6	102.4 ± 24.5	75.3 ± 16.3	62.6 ± 11.0	n/d
U6	75.4 ± 11.8	64.4 ± 2.9	70.8 ± 4.5	65.4 ± 7.3	61.7 ± 9.5	105.8 ± 21.3	93.8 ± 24.5	78.9 ± 15.3	76.7 ± 10.4	68.6 ± 7.9	n/d

Values are mean ± SE of at least three independent experiments. Statistical analysis was by ANOVA followed by Dunnett's test. * Denotes significant difference ($P<0.05$) in cell proliferation relative to untreated control HepG2 cells. n/d: IC_{50} was not determinable for the range of concentrations tested. U1 is a 30kDa Retentate prepared via alkaline hydrolysis and subjected to further hydrolysis by CorPPFla. U2 is a 30kDa Retentate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcFla. U3 is a 30kDa Permeate prepared via alkaline hydrolysis and subjected to further hydrolysis by CorPPFla. U4 is a 30kDa Permeate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcFla. U5 is a 10kDa Permeate prepared via alkaline hydrolysis and subjected to further hydrolysis by CorPPFla. U6 is a 10kDa Permeate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcFla.

Table 11: Effects of brewers' spent grain (BSG) protein hydrolysates A-J (0-0.2% (w/v)) on cell proliferation in the HepG2 cells as measured by (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

	0.005	0.01	0.02	0.025	0.04	0.06	0.08	0.1	0.15	0.2	IC_{50}
A	78.2±0.5	72.6±6.1	71.4±5.1	66.1±7.9	68.7±3.8	70.8±9.4	70.8±3.2	37.3±15.8*	24.1±8.2*	46.4±30.4	0.1
B	84.5±9.2	87.7±12.9	76.4±3.3	76.1±7.4	73.3±2.1	78.8±5.3	80.8±9.4	54.5±16.8	50.0±30.7	47.8±29.0	0.2
C	81.5±0.2	104.7±17.9	88.2±2.1	88.0±8.0	123.7±2.2	91.3±0.3	89.3±8.1	96.2±8.4	104.7±12.3	104.7±7.5	n/d
D	101.5±3.3	84.1±3.6	82.5±6.0	75.6±17.9	83.9±14.7	88.3±9.3	88.0±7.9	109.6±15.3	107.8±9.6	113.7±4.1	n/d
E	70.1±11.4	65.5±6.8	64.9±10.3	61.1±14.0	57.3±7.3	62.8±10.3*	63.7±6.0	68.3±11.9	64.3±5.9	69.9±18.3	n/d
F	73.8±9.6	74.8±10.2	62.1±4.9*	64.6±11.9	65.9±11.2	68.9±11.6	73.9±8.6	73.0±9.6	78.2±6.2	82.5±8.4	n/d
G	92.7±11.3	76.1±7.6	58.2±1.4*	60.8±7.6*	66.1±6.9*	69.1±5.6*	69.5±2.8*	70.6±9.1	78.5±4.5	80.9±15.6	n/d
H	76.0±10.8	67.5±7.4	61.2±5.4	62.5±8.2	62.0±9.8	86.3±22.6	66.0±3.6	71.7±4.5	72.0±6.9	83.1±2.4	n/d
I	94.8±2.6	98.8±6.6	82.5±1.1	90.9±5.6	93.3±10.0	85.6±2.0	95.3±5.1	69.3±21.5	54.1±32.4	59.7±37.4	0.24
J	78.0±9.4	105.5±30.5	75.7±3.0	81.5±10.4	72.7±6.0	79.6±6.8	78.6±4.1	77.0±6.8	79.0±12.0	85.3±12.6	n/d

Values are mean ± SE of at least three independent experiments. Statistical analysis was by ANOVA followed by Dunnett's test. * Denotes significant difference ($P<0.05$) in cell proliferation relative to untreated control HepG2 cells. n/d: IC_{50} was not determinable for the range of concentrations tested. A is unhydrolysed BSG protein. B is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Brewers Clarex and Flavourzyme. C is BSG protein treated with Depol 740L, Brewers Clarex and Flavourzyme. D is BSG protein treated with Shearzyme, Ultraflo Max, Brewers Clarex and Flavourzyme. E is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Alcalase and Flavourzyme. F is BSG protein treated with Depol 740L, Alcalase and Flavourzyme. G is BSG protein treated with Shearzyme, Ultraflo Max, Alcalase and Flavourzyme. H is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. I is BSG protein treated with Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. J is BSG protein treated with Shearzyme, Ultraflo Max, Alcalase, Brewers Clarex and Flavourzyme.

Table 12: Effects of brewers' spent grain (BSG) protein hydrolysates A1-E4 (0-0.2% (w/v)) on cell proliferation in the MCF-7 cells as measured by (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

	0.005	0.01	0.02	0.025	0.04	0.06	0.08	0.1	0.15	0.2	IC_{50}
A1	83.8±4.0	80.1±3.1	84.4±9.1	79.9±5.8	85.9±7.1	83.1±5.8	76.4±5.6	73.9±6.3	60.4±10.1*	39.0±10.7*	0.26
S1	80.4±12.9	73.8±5.7	71.8±8.4	67.4±5.5*	64.4±3.0*	68.1±10.0*	66.0±4.8*	61.1±7.2*	75.0±5.1	71.0±7.3	n/d
E1	78.9±1.1	80.1±9.4	72.8±5.5	73.6±12.8	71.6±8.9	76.9±10.5	72.4±6.5	79.0±13.9	75.3±15.8	76.8±12.9	n/d
A2	86.1±5.5	86.3±4.0	82.9±7.6	80.2±4.0	91.8±4.5	80.3±9.8	67.8±10.4*	62.0±9.5*	43.6±1.3*	50.2±12.0*	0.18
S2	75.8±7.6*	71.8±1.7*	73.5±1.1*	64.1±6.2*	71.4±4.8*	66.4±6.3*	75.4±2.4*	80.6±5.3*	79.5±5.2*	77.7±3.5*	n/d
E2	93.6±17.3	91.0±12.2	96.7±14.5	91.0±13.9	82.9±10.5	83.4±11.3	84.4±12.0	91.0±13.4	88.4±22.4	98.2±16.9	n/d
A3	86.0±14.7	96.0±15.9	86.7±15.4	85.9±10.0	88.7±7.6	88.5±1.9	60.7±18.5	81.8±10.2	57.3±6.4	59.0±8.2	0.31
S3	80.0±3.8	73.5±7.5*	72.9±3.4*	68.6±1.3*	83.1±7.2	79.8±7.4	82.4±6.8	73.5±7.1*	75.3±6.9*	74.2±5.7*	n/d
E3	91.2±5.0	87.3±7.4	84.6±3.4	84.1±3.4	79.1±0.4	83.9±6.1	93.1±14.0	84.2±5.9	91.7±13.8	93.9±9.2	n/d
A4	90.5±18.7	93.2±24.7	89.8±22.5	93.0±12.7	106.1±14.5	116.7±21.0	100.2±4.5	96.5±10.3	62.3±9.6	32.4±8.1*	0.17
S4	85.6±2.0	101.4±2.9	97.3±2.8	95.7±1.9	95.3±2.7	87.9±5.6	81.3±7.1	96.5±1.4	84.0±10.6	83.1±8.9	n/d
E4	94.0±9.1	92.0±18.9	90.9±23.4	81.6±19.6	91.7±6.1	89.7±5.9	81.2±15.3	92.9±20.5	95.6±0.2	96.0±14.7	n/d

Values are mean ± SE of at least three independent experiments. Statistical analysis was by ANOVA followed by Dunnett's test. * Denotes significant difference ($P<0.05$) in cell proliferation relative to untreated control MCF-7 cells. n/d: IC_{50} was not determinable for the range of concentrations tested. A1 is a BSG protein hydrolysate prepared via alkaline hydrolysis and subjected to further hydrolysis by ProPro. S1 is A1 subjected to SGID. E1 is prepared via direct enzyme hydrolysis by ProPro. A2 is a BSG protein hydrolysate prepared via alkaline hydrolysis and subjected to further hydrolysis by CorPPFla. S2 is A2 subjected to SGID. E2 is prepared via direct enzyme hydrolysis by CorPPFla. A3 is a BSG protein hydrolysate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcPro. S3 is A3 subjected to SGID. E3 is prepared via direct enzyme hydrolysis by AlcPro. A4 is a BSG protein hydrolysate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcFla. S4 is A4 subjected to SGID. E4 is prepared via direct enzyme hydrolysis by AlcFla.

Table 13: Effects of brewers' spent grain (BSG) protein hydrolysates U1-U6 (0-0.2% (w/v)) on cell proliferation in the MCF-7 cells as measured by (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

	0.005	0.01	0.02	0.025	0.04	0.06	0.08	0.1	0.15	0.2	IC_{50}
U1	100.1±4.1	101.3±1.3	94.9±2.6	105.0±7.2	90.8±3.6	96.0±14.4	97.4±5.3	111.0±1.7	61.4±12.1	35.9±20.7*	0.17
U2	99.2±11.2	92.4±11.2	93.1±7.9	101.3±5.7	104.6±1.9	93.3±4.1	94.8±18.1	88.8±3.9	93.0±10.8	93.5±19.0	n/d
U3	89.8±2.5	87.9±11.5	87.3±2.7	94.6±12.6	81.0±4.5	71.1±7.5	59.9±19.8	72.0±10.4	59.2±5.9	60.6±11.4	0.3
U4	98.8±2.8	96.0±4.0	93.2±4.2	98.2±14.0	83.3±11.1	97.8±12.6	90.5±12.2	94.4±18.3	62.3±13.0	67.1±10.5	0.29
U5	92.0±3.9	83.6±2.4	86.6±1.5	94.6±12.8	93.6±12.8	87.8±1.5	77.6±1.0	80.6±4.4	79.3±2.9	70.7±3.8*	2.2
U6	96.9±9.6	83.0±6.6	74.3±10.3	80.5±1.5	77.1±0.6	82.6±4.0	83.6±4.4	83.4±0.9	65.1±11.8*	77.7±5.6	n/d

Values are mean ± SE of at least three independent experiments. Statistical analysis was by ANOVA followed by Dunnett's test. * Denotes significant difference ($P<0.05$) in cell proliferation relative to untreated control MCF-7 cells. n/d: IC_{50} was not determinable for the range of concentrations tested. U1 is a 30kDa Retentate prepared via alkaline hydrolysis and subjected to further hydrolysis by CorPPFla. U2 is a 30kDa Retentate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcFla. U3 is a 30kDa Permeate prepared via alkaline hydrolysis and subjected to further hydrolysis by CorPPFla. U4 is a 30kDa Permeate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcFla. U5 is a 10kDa Permeate prepared via alkaline hydrolysis and subjected to further hydrolysis by CorPPFla. U6 is a 10kDa Permeate prepared via alkaline hydrolysis and subjected to further hydrolysis by AlcFla.

Table 14: Effects of brewers' spent grain (BSG) protein hydrolysates A-J (0-0.2% (w/v)) on cell proliferation in the MCF-7 cells as measured by (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

	0.005	0.01	0.02	0.025	0.04	0.06	0.08	0.1	0.15	0.2	IC_{50}
A	71.5±1.0*	71.1±13.2*	61.4±5.9*	58.0±2.3*	59.7±0.2*	60.0±1.8*	60.0±1.4*	63.6±2.6*	69.9±0.9*	72.9±2.6*	n/d
B	70.9±6.2	82.6±18.0	65.0±3.0*	61.2±5.4*	63.5±2.4*	59.6±0.5*	58.7±0.2*	64.8±0.5*	61.7±5.4*	70.0±5.0*	n/d
C	76.8±9.1	84.6±9.2	86.1±15.5	71.0±11.4	78.6±23.2	81.8±23.4	86.8±15.0	77.9±9.9	86.8±20.3	85.3±14.4	n/d
D	79.7±1.3	93.3±19.6	77.6±5.5	74.5±10.4	74.3±3.4	69.5±2.1	69.9±2.7	81.1±0.1	77.4±2.1	81.6±6.6	n/d
E	90.0±23.2	88.7±20.7	90.0±19.2	70.9±13.5	79.7±21.0	81.6±18.6	81.7±16.2	83.9±19.0	88.4±16.4	95.6±12.0	n/d
F	85.3±10.4	70.4±8.6	67.0±9.6	70.3±8.3	78.8±10.7	73.7±8.1	79.0±13.1	83.1±9.2	92.2±15.7	91.7±9.0	n/d
G	79.4±12.1	72.9±8.3	65.4±9.3	67.0±4.5	69.8±9.5	68.9±8.2	70.6±8.3	76.9±7.6	74.5±8.2	78.5±9.9	n/d
H	81.8±3.4	61.7±14.8*	57.5±14.6*	53.9±6.2*	63.9±2.6	61.1±4.5*	77.3±10.5	59.9±0.9*	66.4±2.1	75.6±9.0	n/d
I	70.3±3.2	78.6±5.9	76.7±9.8	82.8±12.8	68.3±5.2	84.4±8.7	84.5±7.0	84.9±8.9	85.7±13.0	105.2±15.0	n/d
J	92.0±15.6	80.7±14.0	76.8±11.1	85.9±15.9	78.8±8.4	90.2±16.1	74.9±4.4	95.8±9.9	92.9±14.0	119.6±23.4	n/d

Values are mean ± SE of at least three independent experiments. Statistical analysis was by ANOVA followed by Dunnett's test. * Denotes significant difference ($P<0.05$) in cell proliferation relative to untreated control MCF-7 cells. n/d: IC_{50} was not determinable for the range of concentrations tested. A is unhydrolysed BSG protein. B is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Brewers Clarex and Flavourzyme. C is BSG protein treated with Depol 740L, Brewers Clarex and Flavourzyme. D is BSG protein treated with Shearzyme, Ultraflo Max, Brewers Clarex and Flavourzyme. E is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Alcalase and Flavourzyme. F is BSG protein treated with Depol 740L, Alcalase and Flavourzyme. G is BSG protein treated with Shearzyme, Ultraflo Max, Alcalase and Flavourzyme. H is BSG protein treated with Shearzyme, Ultraflo Max, Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. I is BSG protein treated with Depol 740L, Alcalase, Brewers Clarex and Flavourzyme. J is BSG protein treated with Shearzyme, Ultraflo Max, Alcalase, Brewers Clarex and Flavourzyme.

In a similar manner to the effects on Jurkat T cells, alkaline- and enzyme-extracted hydrolysates (A1-A4, E1 and E3) exerted dose-dependent effects on U937 cell proliferation (Table 6). Additionally, as was the case in Jurkat T cells, IC_{50} values could not be determined for SGID-treated hydrolysates (S1-S4), in U937 cells. It is also noteworthy, that IC_{50} values for A1-A4 and E1-E4 were higher in U937 cells compared to Jurkat T cells. Apart from U1 and U2, no significant effects on U937 cell proliferation were noted with membrane fractionation-treated hydrolysates (Table 7). Apart from hydrolysate E, enzyme-extracted hydrolysates (A-J) did not have any significant effects on the proliferation of U937 cells (Table 8).

HepG2 cell proliferation was determined following incubation with BSG protein hydrolysates A1-E4, U1-U6 or A-J (Tables 9-11). Significant ($P<0.05$) decreases in cell proliferation across the concentration range tested, were noted with extracts E2 and A4 (Table 9). Apart from U1, the ultrafiltration-generated hydrolysates did not have a significant impact on HepG2 cell proliferation (Table 10). The overall impact of hydrolysates produced using different enzyme combinations (A-J), on HepG2 cell proliferation, was low (Table 11). Only samples A, E, F and G produced significant ($P<0.05$) decreases in cell proliferation.

MCF-7 cells were exposed to hydrolysates A1-E4 for 24 hours and the effects on cell proliferation were then measured (Table 12). In a similar pattern to Jurkat T cells and U937 cells, alkaline-extracted hydrolysates (A1-A4) demonstrated dose-dependent effects on MCF-7 cells. The IC_{50} values of these hydrolysates were also higher, when compared with corresponding values in Jurkat T cells and U937 cells. Furthermore, as was the case with other cell lines, IC_{50} values for S1-S4 could not be determined. MCF-7 cells treated with U1, U5 and U6 at 0.2%, 0.2% and 0.15% (w/v) respectively, showed significant decreases in cell proliferation (Table 13). Among the enzyme-extracted hydrolysates (A-J) administered to MCF-7 cells, only hydrolysates A, B and H had significant effects on cellular proliferation (Table 14).

Apoptosis

The IC_{50} values of BSG phenolic extracts in U937 cells were previously determined (Crowley *et al*., 2017 and Table 3, Chapter 3). U937 cells were treated with BSG phenolic extracts (IC_{50} values) or Doxorubicin (0.5 µg mL^{-1}) for 24 hours and the number of apoptotic cells were counted. Figure 1 shows that only extract PW3 and the cancer-treatment drug, Doxorubicin, significantly induced apoptosis in U937 cells. The increase in fragmented DNA (a key feature of apoptosis) is seen in Figure 2.

Discussion

Ethnicity, age, lifestyle and diet are all factors that can have a significant impact on cancer expression and progression (Roleira *et al*., 2015). Excessive proliferation, an inability to initiate apoptosis under normal conditions and an extended or immortalised life span are the main characteristics of malignant cells (Dai and Mumper, 2010). Certain compounds such as phenolics are known to have an ability to halt this progressive proliferation. Any disruption of the cell cycle specific proteins by phenolics may alter the continuous proliferation (Dai and Mumper, 2010). Studies have shown that observed cytotoxic effects of phenolic compounds is generally related to cell cycle arrest proceeded by apoptosis (Roleira *et al*., 2015). Cells undergoing apoptosis can be recognised and removed by phagocytosis (Edinger and Thompson, 2004). Among the morphological features of apoptosis are caspase activation, chromatin condensation and DNA fragmentation (Edinger and Thompson, 2004; Dai and Mumper, 2010). In many studies involving phenolic compounds, correlations are observed between the antiproliferative activity and the antioxidant activity, suggesting that oxidative mechanisms are involved in cancer cell proliferation. Additionally, it has been noted that extracts with a higher phenolic content, tended to demonstrate higher antioxidant and cytotoxic activities (Roleira *et al*., 2015).

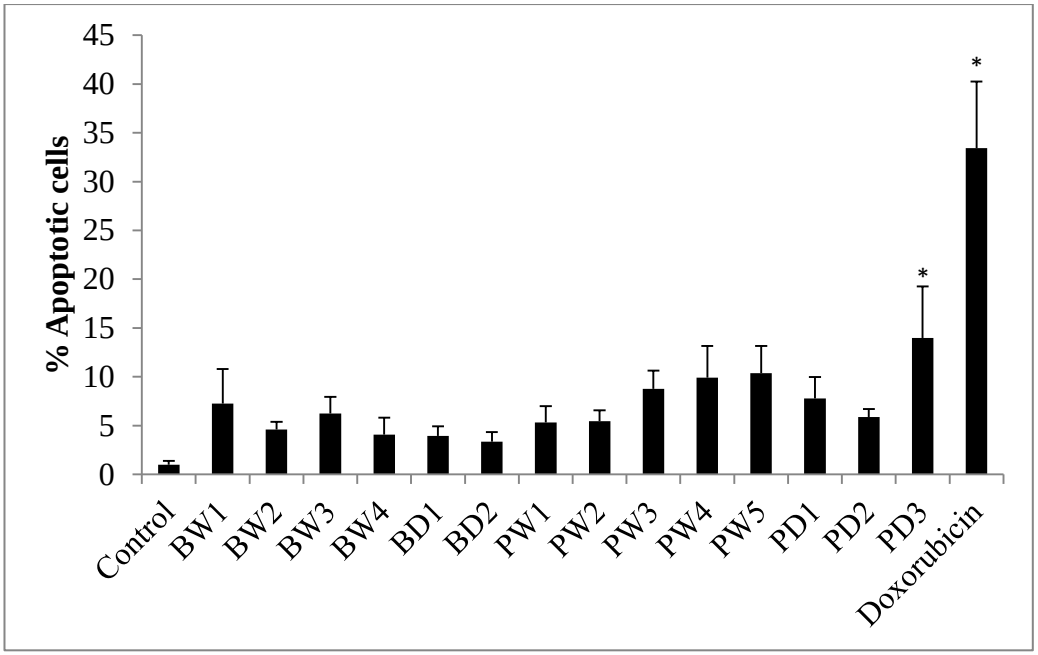


Figure 1: Effect of BSG phenolic extracts (IC_{50} values) or Doxorubicin (0.5 µg mL^{-1}) on apoptosis in U937 cells. BW1-4 represent extracts from black, wet BSG; BD1-2 represents extracts from black, dry BSG; PW1-5 represents extracts from pale, wet BSG and PD1-3 represents extracts from pale, dry BSG. Cells were treated with BSG extracts or Doxorubicin for 24 hours and were then harvested by centrifugation and stained with Hoechst 33342. 300 cells were examined at 200X magnification, and the percentage of condensed and fragmented nuclei was calculated. Values are mean ± SE of at least three independent experiments. Statistical analysis by ANOVA followed by Dunnett's Test. * Denotes statistically significant difference in apoptosis, relative to untreated U937 cells (control) ($P<0.05$).

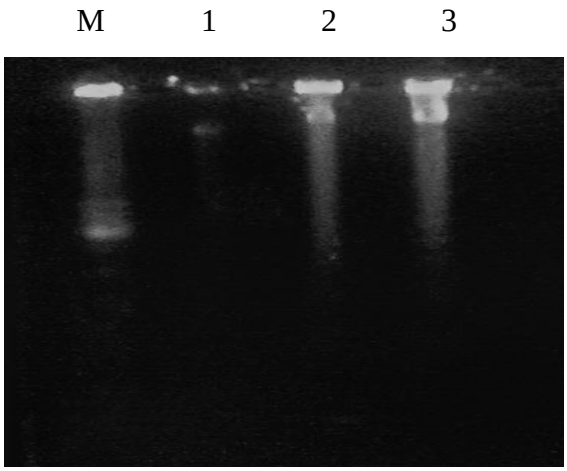


Figure 2: Apoptotic potential of BSG phenolic extract PD3 and Doxorubicin in U937 cells (DNA fragmentation). Lane M: 100– 1500 bp DNA standard. Lane 1: control (untreated cells). Lane 2: PD3 (45% v/v). Lane 3: Doxorubicin (0.5 µg mL^{-1}).

Here, the antiproliferative potential of phenolic extracts and protein hydrolysates from BSG were assessed in the cancer cell lines; Jurkat T cells, RAW 264.7 cells, MCF-7 cells, U937 cells and HepG2 cells. The antiproliferative effects of the BSG samples were assessed using the MTT assay in all cell lines, while the apoptotic potential of BSG phenolic extracts were measured in U937 cells using the Hoechst staining and DNA fragmentation assays. The MTT assay measures the reduction of yellow tetrazolium salt (MTT) by the mitochondrial enzymes of metabolically active cells resulting in the formation of an insoluble purple product known as formazan. The number of living cells is directly proportional to the amount of formazan product formed. Results are expressed as a percentage compared to the control cells not exposed to test compounds (Prabhakar *et al*., 2015). The measurement of apoptosis induction in cancer cell lines is a valid indication of a compound's potential to perform as a chemotherapeutic agent (Kenny *et al*., 2013). The DNA fragmentation assay entails the isolation of apoptotic DNA fragments via lysing the isolated nuclei of apoptotic cells using a detergent such as NP-40. Apoptotic DNA fragments released can be separated by gel electrophoresis (Prabhakar *et al*., 2015).

The BSG phenolic extracts assessed here were produced using an enzyme-extraction method previously outlined by Crowley *et al*. (2017) and described in Chapter 3. The extracts were assessed for their antiproliferative potential in Jurkat T cells, RAW 264.7 cells and MCF-7 cells (Tables 1-3). Dose-dependent decreases in Jurkat T cell proliferation were noted with black phenolic extracts (Table 1). McCarthy *et al*. (2014) similarly reported that alkaline-extracted BSG phenolic extracts had a concentration-dependent effect on Jurkat T cell proliferation. Results presented here, demonstrated that IC_{50} values for black phenolic extracts were likewise low when compared with IC_{50} values previously reported in another suspension cell line; U937 cells (Crowley *et al*., 2017). The IC_{50} values of pale BSG phenolic extracts assessed here were higher than values reported with BSG phenolic extracts produced using alkaline hydrolysis (McCarthy *et al*., 2014).

The adherent cell lines, RAW 264.7 cells and MCF-7 cells were also used to measure the impact of phenolic extracts on cell proliferation. Phenolic extracts were more toxic in RAW 264.7 cells compared to MCF-7 cells, as indicated by

the lower IC_{50} values (Tables 2-3). This contrast may be explained by their difference in sensitivity to the extracts and the specific origin of the cells; RAW 264.7 cells are a murine macrophage cell line, while MCF-7 cells are derived from human metastatic breast tissue. Melo *et al*. (2015) identified a number of phenolic compounds in winery by-products and reported that extracts from Petit Verdot pomace and Chenin Blanc rachis significantly decreased RAW 264.7 cell proliferation at higher concentrations. Different parts of the *Jatropha curcas* plant were assessed for their cytotoxic effects in RAW 264.7 cells (Othman *et al*., 2015). The authors found that almost 95% of the cell growth was inhibited by a crude extract from the plant root, while a hexane partition from the plant also inhibited cell growth. Terpenoids were identified in the partition, which the authors note have been reported to show cytotoxic effects towards animal cells lines. A number of other studies have utilised MCF-7 cells to assess the cytotoxic effects of plant extracts (Lopez *et al*., 2013; Ahmed *et al*., 2016; Parra Pessoa *et al*., 2016). Parra Pessoa *et al*. (2016) reported that ethanolic seed extracts from *Licania rigida* and *Licania tomentosa* were not cytotoxic to MCF-7 and Caco-2 cells.

Protein hydrolysates from BSG were also assessed in cancer cell lines (Tables 4-14). Treatment of Jurkat T cells with alkaline-extracted hydrolysates and enzyme-extracted hydrolysates led to a dose-dependent decrease in cell proliferation (Table 4). McCarthy *et al*. (2013b) noted a similar pattern in Jurkat T cells with alkaline-extracted BSG protein hydrolysates, where an increase in hydrolysate concentration led to a decrease in cell proliferation. BSG protein hydrolysates subjected to SGID (S2-S4) did not have any significant impact on Jurkat T cells. In another study utilising suspension cells, Wang *et al*. (2008) showed that soy protein hydrolysate produced by SGID inhibited L1210 leukemia cell growth in a dose-dependent manner. The BSG protein hydrolysates (A1-E4) had a lesser impact on U937 cell proliferation (Table 6) compared with Jurkat T cells, as seen with the higher IC_{50} values. Alkaline-extracted BSG protein hydrolysates (A1-A4) had a dose-dependent effect on U937 cell proliferation (Table 6). A similar pattern on U937 cell proliferation was noted by McCarthy *et al*. (2013a; 2013b), using a different set of alkaline-extracted BSG protein hydrolysates. U937 cells were previously used to show the antiproliferative

activity of peptides derived from anchovy sauce (Lee *et al*., 2004), where results showed that the peptide fraction induced apoptosis in U937 cells.

In HepG2 cells, IC_{50} values could not be calculated for hydrolysates A1-E4 (Table 6). Out of the BSG protein hydrolysates subjected to ultrafiltration, only the 30kDa retentate produced by Corolase PP and Flavourzyme (U1) demonstrated significant toxic effects (Table 7). The 10kDa permeate produced using Alcalase and Flavourzyme (U6) did not have any significant impact on HepG2 cell proliferation. In another report, BSG protein hydrolysates generated using brewers' spent yeast (BSY) proteases, Neutrase and Alcalase, were assessed for their effects on HepG2 cell proliferation (Vieira *et al*., 2017). The < 10 kDa fraction produced using Alcalase significantly decreased cell proliferation, whereas the BSY< 10 kDa fraction significantly increased proliferation. The antiproliferative potential of other low molecular weight food peptide fractions has previously been reported. Rice bran was used to generate bioactive GI-resistant peptides, resulting in <5 and 5-10 kDa fractions, which were able to inhibit the growth of HepG2 cells more effectively than the non-resistant peptide fractions (Kannan *et al*., 2008). Peptide fractions (<10 and 10-30kDa) from shrimp shell whites and langostino shells significantly inhibited the growth of HepG2 cells (Kannan *et al*., 2011). Low molecular weight fractions (< 3 kDa) of loach protein hydrolysates were found to be more effective at inhibiting the HepG2 cell proliferation than higher molecular weight fractions (You *et al*., 2011).

The antiproliferative effects of BSG protein hydrolysates were also assessed in MCF-7 cells (Tables 12-14). A dose-dependent decrease in MCF-7 cell proliferation was seen with alkaline-extracted BSG protein hydrolysates (A1-A4) (Table 12). Ma *et al*. (2015) showed that bioactive peptides in walnut residual protein were released during papain hydrolysis and had a dose-dependent cytotoxic effect in MCF-7 cells. The purified peptide had an amino acid sequence of CTLEW with a molecular weight of 651.2795 Da. The 30kDa retentate (U1) and 10kDa permeates U5 and U6, demonstrated cytotoxic effects at higher concentrations in MCF-7 cells (Table 13). It was found that a loach protein hydrolysate and its <3kDa fraction significantly inhibited the proliferation of viable MCF-7 cells compared to the higher molecular weight fractions, with lower

levels of proliferation seen at higher hydrolysate concentrations (You *et al*., 2011).

The apoptotic effects of BSG phenolic extracts were further assessed in U937 cells (Figures 1-2). U937 cells have been widely used in apoptosis research and were therefore chosen for this investigation. The BSG phenolic extract, PD3, at 45% (v/v), was the only BSG sample able to significantly increase the number of apoptotic U937 cells (Figure 1). The cancer treatment drug, Doxorubicin, also increased the number of apoptotic cells to 33.4%. The proapoptotic effects of Doxorubicin have already been demonstrated in U937 cells (Kenny *et al*., 2013). Apoptosis is a form of programmed cell death, where unwanted cells are removed from the body (Prabhakar *et al*., 2015). Cells that have undergone apoptosis typically show chromatin condensation and DNA fragmentation (Dai and Mumper, 2010). In the DNA fragmentation assay, the extract produced the characteristic laddering appearance of apoptotic cells noted in Figure 2. The absence of proapoptotic effects of the other phenolic extracts may be explained by the lower IC_{50} values used. Extract PD3 has previously demonstrated cellular antioxidant ability, protecting against H_2O_2-induced DNA damage in U937 cells and H_2O_2-induced decreases in glutathione content and superoxide dismutase activity in HepG2 cells (Crowley *et al*., 2017 and Figures 1-3, Chapter 3). Therefore, the apoptotic effect here may be related to its antioxidant activity. It has previously been shown that ferulic acid is the major hydroxycinnamic acid of BSG (McCarthy *et al*., 2013c). The apoptotic effects of ferulic acid have previously been demonstrated in HepG2 cells by Prabhakar *et al*. (2015). Ferulic acid demonstrated apoptotic potential as evidenced by cell shrinkage, cell budding, discrete nuclei fragments, Bax upregulation and Bcl-2 downregulation. Park *et al*. (2008) showed that the phenolic compound, esculetin, induced apoptosis in U937 cells via Bcl-2 regulation and caspase activity. Further investigation is required to confirm the exact molecular mechanisms involved in the apoptotic effects of PD3. Also the specific components within the extract responsible for the apoptotic effects will need to be identified.

Conclusions

To conclude, BSG phenolic extracts demonstrated greater antiproliferative ability in cell lines, compared with BSG protein hydrolysates. In general, the suspension cell lines, Jurkat T cells and U937 cells, displayed a greater sensitivity to the BSG extracts. Similarly, with regard to the BSG phenolic extract, the animal-derived RAW 264.7 cells were more sensitive to extracts compared to the human breast cancer cell line, MCF-7. The black BSG phenolic extracts generally displayed greater antiproliferative activity in cells compared to extracts derived from pale BSG. This may be due to Maillard reaction products generated during the roasting of barley grains. However, the apoptotic effects of BSG phenolic extracts were, in general, found to be low, suggesting that apoptosis is not the primary mechanism by which the extracts exert their antiproliferative effects.

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Chapter 9

General Discussion

Cereal grains are produced by plants of the grass family Poaceae. These include wheat, triticale, rye, barley, oats, maize, rice, sorghum and millet (Wrigley, 2010). Cereal grains are known to be a source of macronutrients, minerals and vitamins (Carnevali *et al*., 2014). Additionally, cereal grains possess a number of biologically active substances such as arabinoxylans (AX), β-glucans, cellulose, lignans, lignin, sterols, tocopherols, tocotrienols, alkylresorcinols, phenolic acids and microelements (Bartlomiej *et al*., 2012). Barley constitutes approximately 5 to 8 percent of world cereal production and is favoured for beer production with wheat flour commonly used as an adjunct (Batey, 2010; Edney, 2010). Brewers' spent grain (BSG) is the insoluble part of the barley grain remaining after the mashing step in the brewing process. BSG consists of the seed coat–pericarp–husk layers that encased the original barley grain (Lynch et al., 2016). Owing to the large quantities produced annually, current low market value, growing environmental awareness and the appreciation that BSG is a nutritionally valuable co-product, further uses regarding the health benefits of BSG are currently being investigated (Lynch et al., 2016).

The purpose of this research was to investigate the potential health benefits of the phenolic and protein components of BSG, using *in vitro* cell culture models. Previous research on BSG had assessed the bioactivity of phenolic extracts and protein hydrolysates derived from BSG using an alkaline extraction procedure. The phenolic extracts and protein hydrolysates examined here were generated using an extraction procedure utilising food-grade carbohydrase and proteinase enzymes. Alkaline-extracted phenolic and protein fractions from BSG have previously demonstrated antioxidant and anti-inflammatory activity. Enzyme extraction may offer an alternative to common solvent extraction procedures and owing to their natural origin may be more acceptable for use in functional food products.

The first experimental chapter (Chapter 2) in this thesis examined a BSG protein-enriched isolate extracted using an alkaline extraction method and hydrolysed using Alcalase (U) and 3 fractions prepared from U using membrane fractionation; a 5 kDa retentate (U >5), a 5kDa permeate (U <5) and a 3 kDa permeate (U <3), which were added to low-fat milk. The anti-inflammatory potential of these hydrolysates was previously reported in McCarthy *et al*.

(2013a). The supplemented milk samples were then subjected to simulated gastrointestinal digestion (SGID) and the anti-inflammatory potential of the resultant digestates was investigated in concanavalin-A (ConA)-stimulated Jurkat T cells and lipopolysaccharide (LPS)-stimulated murine macrophage RAW 264.7 cells. Interleukin 6 (IL-6) production was significantly decreased ($P<0.05$) in stimulated Jurkat T cells in the presence of BSG protein hydrolysate-supplemented milk digestates, with no apparent effect on interferon gamma (IFN-γ) or IL-2 secretion. The BSG protein hydrolysate, U, and its 5kDa retentate (U >5), were previously shown to have anti-inflammatory effects, by reducing the production of IFN-γ in ConA-stimulated Jurkat T cells, when added directly to cells (McCarthy *et al*., 2013a). This may indicate that the concentration of BSG hydrolysates added to milk may need to be increased in order to elicit a significant effect on IFN-γ production. None of the digested milk samples had a significant effect on the production of IL-6, IL-1β or tumor necrosis factor alpha (TNF-α) in LPS-stimulated RAW 264.7 cells, possibly highlighting a cell specific effect. Functional foods containing bioactive ingredients need to demonstrate an ability to retain their bioactivity following digestion. A number of factors, including the food matrix chosen, can impact on the anti-inflammatory activity of BSG protein hydrolysates following their addition into a food vehicle and subjection to gastrointestinal digestion. However, further research, including additional inflammatory markers, is necessary to optimise the anti-inflammatory effect of BSG hydrolysate-supplemented food products. One particular challenge to the commercialisation of bioactive protein hydrolysates is the lack of information available regarding their bioavailability which is the amount of an ingested nutrient that is absorbed and metabolised (Li-Chan, 2015).

Enzyme extraction using cell wall degrading enzymes was utilised to generate phenolic compounds from both black and pale BSG. Black BSG, from dark wort production, is produced following the roasting of barley grain at 200°C. Pale BSG is obtained from the standard brewing process (Connolly *et al*., 2013; Piggott *et al*., 2014). Hydroxycinnamic acids (HCAs) are the principal class of phenolic compounds found in BSG, with ferulic acid being present in the highest amounts (McCarthy *et al*., 2013b). HCAs are known for their antioxidant activity and research has shown that HCAs can be used as preventive and/or therapeutic agents

in several diseases related to oxidative stress including atherosclerosis, inflammatory injury, cancer and cardiovascular diseases (Teixeira *et al*., 2013). The DNA protective effects of BSG phenolic extracts were measured by their ability to protect against hydrogen peroxide (H_2O_2)-induced DNA single strand breaks in U937 cells using the comet assay (Chapter 3). The effects of BSG phenolic extracts on the cellular antioxidant status in HepG2 was assessed by measuring the cellular content of glutathione (GSH) and the activity of the enzymes superoxide dismutase (SOD) and catalase (CAT) following an oxidative challenge. Phenolic extracts protected against DNA damage however, the use of enzymes in the extraction procedure did not significantly enhance the DNA protective effects of the extracts. The extracts prepared using enzymes were more effective in protecting against a depletion of cellular antioxidant GSH content and SOD and CAT activity in oxidant-challenged HepG2 cells than the extracts prepared without enzymes. This study indicates that enzyme-extraction could be used in the production of novel, natural, plant-derived bioactive components for use in the food industry as an alternative to conventional solvent extraction. However, it will be necessary to fully characterise the bioactive constituents of the extracts however – particular with regard to the black BSG phenolic extracts, to elucidate the exact bioactive components responsible for the noted antioxidant effects. Also of consideration for future research, is the impact of phenolic extracts on other cell lines such as intestinal cells. This may be of greater relevance considering the route these compounds would take during the digestive process and would also provide a useful cell culture model for bioavailability studies. Another important consideration is the *in vivo* levels of phenolic extracts following absorption. The concentrations used in cell lines in this study may not be physiologically relevant considering the reported poor bioavailability of phenolic compounds (Manach *et al*., 2005). For example, ferulic acid has poor water solubility and hence poor bioavailability (Mateo Anson *et al*., 2009).

Following this, the ability of enzyme-extracted BSG phenolic extracts to enhance the antioxidant potential of flavoured water drinks before and after *in vitro* digestion was investigated (Chapter 4). *In vitro* digestion models offer a truer reflection of the health benefits of bioactive compounds, revealing the amount of a compound available for absorption. The addition of phenolic extracts

to water drinks did not significantly increase antioxidant activity. The lack of an enhancement effect with fortification of the drinks may be related to the low extract concentration (10% v/v) added to the water drinks. The antioxidant effects of the phenolic extracts are likely to have been diluted. Another issue noted here was the apparent discrepancy between the antioxidant methods used (DPPH, FRAP and ORAC). These conflicting results may, in future, be avoided by applying the relative antioxidant capacity index (RACI), which allows for the accurate ranking of the antioxidant capacity of foods (Sun and Tanumihardjo, 2007). It may also be worth exploring alternative uses for BSG phenolic extracts, outside of human nutrition. For example, certain plant extracts are known to have anti-fungal activity (Kalidindi *et al*., 2015; Sales *et al*., 2016). Owing to their natural origin and hence environmental safety, phenolic extracts from BSG may have potential phytosanitary applications.

The potential of using carbohydrase (Shearzyme, Ultraflo Max and Depol 740L) and proteinase (Alcalase, Brewers Clarex and Flavourzyme) enzymes to extract BSG protein hydrolysates with anti-inflammatory and antioxidant activities, was also assessed (Chapter 5). The impact of BSG protein hydrolysates on cytokine production in both ConA-treated Jurkat T cells and LPS-stimulated RAW 264.7 cells, DNA damage in H_2O_2-treated U937 cells and GSH content, SOD and CAT activity of H_2O_2-treated HepG2 cells was assessed. The carbohydrase combination of Shearzyme and Ultraflo Max and proteinase combination of Alcalase, Brewers Clarex and Flavourzyme was the most effective enzyme treatment yielding bioactive BSG hydrolysates. The unhydrolysed fraction also demonstrated both anti-inflammatory and antioxidant activity, indicating that other components present in the BSG may be responsible for its bioactivity. Further research is needed to fully elucidate the molecular mechanisms involved and the specific amino acid sequences that are responsible for the biological activity. The use of animal studies and clinical trials would also uncover any potentially negative effects associated with bioactive peptides (Santiago-Lopez *et al*., 2016). This is a necessary step in the development of more effective functional food products targeting specific health issues.

The anti-inflammatory activity and antioxidant potential of hydrolysates generated using direct enzymatic hydrolysis procedure was investigated and

compared with hydrolysates produced using an alkaline-extraction method, alkaline-extracted hydrolysates subjected to SGID and alkaline-extracted hydrolysates generated using ultrafiltration (Chapter 6). Alkaline hydrolysis was found to be more effective than direct enzyme hydrolysis at producing bioactive BSG protein hydrolysates. Both alkaline-extracted BSG protein hydrolysates and alkaline-extracted BSG protein hydrolysates subjected to ultrafiltration, demonstrated both anti-inflammatory and antioxidant activity. Again, however, it will be necessary to characterise the specific bioactive components which are responsible for the noted activity. Considering the market that exists for functional food products aimed at reducing the effects of many chronic diseases, greater emphasis should be put on using relevant purifaiction and characterisation methods to fully identify the bioactive constituents of BSG. This may be achieved by methods such as affinity chromatography and gel filtration chromatography, which have been successfully used to purify and characterise peptides from chickpea protein hydrolysates (Torres-Fuentes *et al*., 2011).

Oxidative stress can lead to the production of ROS and free radicals with potential harmful effects on neuronal cells (Uttara *et al*., 2009). Antioxidant compounds can elicit both neuroprotective (preventing apoptosis) and neuroregenerative effects, through the reduction or reversal of cellular damage and by delaying the progress of neuronal cell loss (Pacifico *et al*., 2014). The neuroprotective potential of BSG phenolic extracts was investigated in SK-N-BE(2) cells treated with H_2O_2 (Chapter 7). It was found that pale BSG extracts protected against the H_2O_2-induced toxicity in SK-N-BE(2) cells, while extracts from both black and pale BSG significantly decreased the number of apoptotic cells. Black and pale BSG extracts protected against the H_2O_2-induced increase in lipid peroxidation. These results suggest that BSG phenolic fractions may have potential as functional food components to prevent and treat neurodegenerative diseases. There is a need to characterise the specific neuroprotective mechanisms of BSG phenolic extracts.

The final segment of this research involved the assessment of the anti-proliferative potential of phenolic extracts and protein hydrolysates from BSG in cell lines from a cancerous origin (Chapter 8). Additionally, the Hoechst staining and DNA fragmentation assays were used to measure the impact of BSG phenolic

extracts on apoptosis in U937 cells. Results indicated that BSG phenolic extracts demonstrated greater antiproliferative ability in cell lines, compared with BSG protein hydrolysates. Additionally phenolic extracts derived from black BSG generally displayed greater antiproliferative activity in cells compared to extracts derived from pale BSG. This may be connected to Maillard reaction products generated during the roasting of barley grains. While BSG phenolic extracts demonstrated antiproliferative effects, the samples' apoptotic effects were, in general, found to be low compared with the cancer treatment drug Doxorubicin, indicating that apoptosis is not the primary mechanism by which the extracts exert their antiproliferative effects.

Conclusions

In conclusion, the research conducted illustrates the potential of BSG as a source of functional food ingredients. Phenolic extracts derived from BSG demonstrated strong antioxidant potential in different cell lines, while enzyme hydrolysis-derived BSG protein hydrolysates showed both anti-inflammatory and antioxidant activity in cellular models. More research needs to be carried out however, in identifying the specific phenolic and protein components that are responsible for the noted bioactivity. Future studies should focus on the inclusion of bioactive extracts into different food types. Additionally, the bioavailability of the bioactive extracts will need to be ascertained, clarifying the ability of these compounds to cross the gut barrier. Alternative uses for BSG bioactive components should also be explored, expanding on the potential human nutrition benefits. Currently, as part of the wider project objectives, a human intervention study is being performed, assessing the impact of BSG extracts on antioxidant and anti-inflammatory biomarkers in healthy human volunteers. Results from this study should add further information as to the potential use of BSG components as effective bioactive food ingredients.

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