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Publications

Research Papers

1. McCarthy AL, O'Callaghan YC, Connolly A, Piggott CO, FitzGerald RJ, O'Brien NM. Phenolic extracts of Brewers' Spent Grain (BSG) as functional ingredients - assessment of their DNA protective effect against oxidant-induced DNA single strand breaks in U937 cells. *Food Chem* (2012) **134**, 641-646.
2. McCarthy AL, O'Callaghan YC, Connolly A, Piggott CO, FitzGerald RJ, O'Brien NM. *In vitro* antioxidant and anti-inflammatory effects of brewers' spent grain protein rich isolate and its associated hydrolysates. *Food Res Int* (2013) **50**, 205-212.
3. McCarthy AL, O'Callaghan YC, Neugart S, Piggott CO, Connolly A, Jansen MAK, Krumbein A, Schreiner M, FitzGerald RJ, O'Brien NM. The hydroxycinnamic acid content of barley and brewers' spent grain (BSG) and the potential to incorporate phenolic extracts of BSG as antioxidants into fruit beverages. *Food Chem* (2013) **141**, 2567-2574.
4. McCarthy AL, O'Callaghan YC, Connolly A, Piggott CO, FitzGerald RJ, O'Brien NM. Anti-inflammatory effects of phenolic enriched fractions from Brewers' Spent Grain (BSG), measured by enzyme-linked immunosorbent assay (ELISA) in the Jurkat T cell line. *J Sci Food Agric* (2013) DOI: 10.1002/jsfa.6421.
5. McCarthy AL, O'Callaghan YC, Connolly A, Piggott CO, FitzGerald RJ, O'Brien NM. Brewers' spent grain (BSG) protein hydrolysates decrease hydrogen peroxide (H₂O₂)-induced oxidative stress and concanavalin-A (con-A) stimulated IFN- γ production in cell culture. *Food Funct* (2013) **4**, 1709-1716.

Review Papers

1. McCarthy AL, O'Callaghan YC, Piggott CO, FitzGerald RJ, O'Brien NM. Brewers' spent grain; bioactivity of phenolic component, its role in animal nutrition and potential for incorporation in functional foods: a review. *Proc Nutr Soc* (2013) **72**, 117-125
2. McCarthy AL, O'Callaghan YC, O'Brien NM. Protein hydrolysates from agricultural crops - bioactivity and potential for functional food development. *Agriculture* (2013) **3**, 112-130
3. McCarthy AL, O'Brien NM. Bioaccessibility of functional ingredients. *Curr Nutr Food Sci* (in press)

Abstracts

1. McCarthy AL, O'Callaghan YC, Piggott CO, FitzGerald RJ, O'Brien NM. Potential of phenolic extracts from Brewer's Spent Grain to protect against oxidant induced DNA damage. *Proc Nutr Soc* (2011) **70**, OCE3.
2. McCarthy AL, O'Callaghan YC, Connolly A, Piggott CO, FitzGerald RJ, O'Connor TP, O'Brien NM. The incorporation of phenolic extracts from brewers' spent grain (BSG) into commercial fruit juices and smoothies. *41st Annual Food Research Conference* (2012) P2.
3. O'Brien NM, McCarthy AL, O'Callaghan YC, Piggott CO, FitzGerald RJ. Bioactivity of protein and phenolic extracts of Brewers' Spent Grain (BSG) - assessment of their DNA protective effect against oxidant-induced DNA single strand breaks in U937 cells. *Proceedings of the 103rd AOCS Annual Meeting and Expo.* (2012) P45.
4. McCarthy AL, O'Callaghan YC, Connolly A, Piggott CO, FitzGerald RJ, O'Brien NM. Phenolic extracts from brewers' spent grain as novel functional food ingredients. *Proceedings of the 2nd Oxford Functional Food Conference* (2012) P56, Abstract ID:8.
5. O'Brien NM, McCarthy AL, O'Callaghan YC, Connolly A, Piggott CO, FitzGerald RJ. Brewers' spent grain (BSG) protein hydrolysates and phenolic co-products: potential use as ingredients in functional foods. *Proceedings of the 104th AOCS Annual Meeting and Expo.* (2013) PCP4.
6. McCarthy AL, O'Callaghan YC, Connolly A, Piggott CO, FitzGerald RJ, O'Brien NM. The antioxidant and anti-inflammatory capacity of phenolic extracts from pale and black brewers' spent grain, measured by *in vitro* cellular methodologies. *42nd Annual Food Research Conference* (2013) P5.

7. McCarthy AL, O'Callaghan YC, Connolly A, Piggott CO, FitzGerald RJ, O'Brien NM. Cellular *in vitro* bioactivity of protein hydrolysates from brewers' spent grain. *Proc Nutr Soc* (2013) OCO41

Abstract

Functional food ingredients, with scientifically proven and validated bioactive effects, present an effective means of inferring physiological health benefits to consumers to reduce the risk of certain diseases. The search for novel bioactive compounds for incorporation into functional foods is particularly active, with brewers' spent grain (BSG, a brewing industry co-product) representing a unique source of potentially bioactive compounds.

The DNA protective, antioxidant and immunomodulatory effects of phenolic extracts from both pale (P1 - P4) and black (B1 - B4) BSG were examined. Black BSG extracts significantly ($P < 0.05$) protected against DNA damage induced by hydrogen peroxide (H_2O_2) and extracts with the highest total phenolic content (TPC) protected against 3-morpholinopyridone hydrochloride (SIN-1)-induced oxidative DNA damage, measured by the comet assay. Cellular antioxidant activity assays were used to measure antioxidant potential in the U937 cell line. Extracts P1 - P3 and B2 - B4 demonstrated significant ($P < 0.05$) antioxidant activity, measured by the superoxide dismutase (SOD) activity, catalase (CAT) activity and glutathione (GSH) content assays. Phenolic extracts P2 and P3 from pale BSG possess anti-inflammatory activity measured in concanavalin-A (conA) stimulated Jurkat T cells by an enzyme-linked immunosorbent assay (ELISA); significantly ($P < 0.05$) reducing production of interleukin-2 (IL-2), interleukin-4 (IL-4, P2 only), interleukin-10 (IL-10) and interferon- γ (IFN- γ). Black BSG phenolic extracts did not exhibit anti-inflammatory effects *in vitro*.

Hydroxycinnamic acids (HA) have previously been shown to be the phenolic acids present at highest concentration in BSG; therefore the HA profile of the phenolic extracts used in this research, the original barley (before brewing) and whole BSG was characterised and quantified using high performance liquid chromatography (HPLC). The

concentration of HA present in the samples was in the order of ferulic acid (FA) > *p*-coumaric acid (*p*-CA) derivatives > FA derivatives > *p*-CA > caffeic acid (CA) > CA derivatives. Results suggested that brewing and roasting decreased the HA content.

Protein hydrolysates from BSG were also screened for their antioxidant and anti-inflammatory potential. A total of 34 BSG protein samples were tested. Initial analyses of samples A – J found the protein samples did not exert DNA protective effects (except hydrolysate H) or antioxidant effects by the comet and SOD assays, respectively. Samples D, E, F and J selectively reduced IFN- γ production ($P < 0.05$) in Jurkat T cells, measured using enzyme linked immunosorbent assay (ELISA). Further testing of hydrolysates K – W, including fractionated hydrolysates with molecular weight < 3, < 5 and > 5 kDa, found that higher molecular weight (> 5 kDa) and unfractionated hydrolysates demonstrate greatest anti-inflammatory effects, while fractionated hydrolysates were also shown to have antioxidant activity, by the SOD activity assay.

A commercially available yogurt drink (Actimel) and snack-bar and chocolate-drink formulations were fortified with the most bioactive phenolic and protein samples – P2, B2, W, W < 3 kDa, W < 5 kDa, W > 5 kDa. All fortified foods were subjected to a simulated gastrointestinal *in vitro* digestion procedure and bioactivity retention in the digestates was determined using the comet and ELISA assays. Yogurt fortified with B2 digestate significantly ($P < 0.05$) protected against H₂O₂-induced DNA damage in Caco-2 cells. Greatest immunomodulatory activity was demonstrated by the snack-bar formulation, significantly ($P < 0.05$) reducing IFN- γ production in con-A stimulated Jurkat T cells. Hydrolysate W significantly ($P < 0.05$) increased the IFN- γ reducing capacity of the snack-bar. Addition of fractionated hydrolysate W < 3 kDa and W < 5 kDa to yogurt also reduced IL-2 production to a greater extent than the unfortified yogurt ($P < 0.05$).

Chapter 1

Literature review

1.1. Introduction

Novel functional food ingredients with proven health benefits are in demand by both the food industry and informed modern consumers. Brewers' spent grain (BSG) is a nutritionally valuable co-product of the brewing process and a potential source of bioactive ingredients. These ingredients include polyphenols, mainly hydroxycinnamic acids, and protein. This literature review aims to evaluate existing knowledge in the area of bioactive phenolic compounds and protein hydrolysates and bioactivity retention following *in vitro* digestion of functional food ingredients. Three individual review papers entitled "Brewers' spent grain – bioactivity of phenolic component, its' role in animal nutrition and potential for incorporation in functional foods; a review", "Protein hydrolysates from agricultural crops — bioactivity and potential for functional food development" and "Bioaccessibility of functional ingredients" have been published in peer-review journals and the literature review will be presented in this format.

Existing evidence regarding BSG is discussed in section 1.2, with a particular focus on the phenolic compounds present in BSG and their extraction, composition and bioactivity. The incorporation of BSG into foods for animal and human nutrition is also reviewed.

Due to the lack of information regarding protein hydrolysates from BSG, a broad review of the bioactivity of protein hydrolysates from a range of agricultural crops was conducted. The bioactive and techno-functional properties associated with protein hydrolysates from agricultural crops are examined in section 1.3.

The ability of a compound to be absorbed across the gastrointestinal tract is critical to its bioactivity. Therefore, the bioaccessibility of functional ingredients is reviewed in section 1.4, including methods used to simulate *in vivo* digestion and factors affecting bioaccessibility. The bioaccessibility of specific functional food ingredients and approaches that can be used to increase bioaccessibility are also discussed.

1.2. Brewers' spent grain – bioactivity of phenolic component, its' role in animal nutrition and potential for incorporation in functional foods; a review.

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1.2.1. Abstract

Brewers' spent grain (BSG) is a low-value co-product of the brewing industry produced in large quantities annually. This paper reviews existing evidence regarding the phenolic component of BSG, focusing on composition, extraction and biofunctions such as antioxidant, anti-atherogenic, anti-inflammatory and anti-carcinogenic activities. Furthermore, the incorporation of BSG in foodstuffs will be discussed, including the use of BSG as an animal feed supplement and the potential of BSG to be incorporated into foods for human consumption. BSG contains hydroxycinnamic acids including ferulic acid, *p*-coumaric acid and caffeic acid; which have shown bioactivity in the pure form (antioxidant, anti-inflammatory, anti-atherogenic, anti-cancer). Phenolic extracts from BSG have also shown antioxidant potential, by protecting against oxidant induced DNA damage, possibly by iron chelation. Studies show that BSG has many benefits when used as a supplement in animal feed, such as increasing milk yield, milkfat content and in providing essential dietary amino acids. The incorporation of BSG in human foods such as cookies and ready-to-eat snacks has resulted in increased protein and fibre contents of the products, where the changes in organoleptic properties are controllable. It can be concluded that the phenolic component of BSG has potential bioactive effects, which are worth pursuing given that the inclusion of BSG into human foodstuffs is viable and beneficial.

1.2.2. Introduction

Brewers' spent grain (BSG) is the solid fraction of barley malt remaining after the production of wort. According to Eurostat Data, BSG is the main by-product of the brewing industry, with approximately 3.4 million tonnes being produced annually in the E.U. (Stojceska *et al.*, 2008), at least 160,000 tonnes of which are produced in Ireland.

This solid residue contains water insoluble proteins in addition to the husk, pericarp and seed coat of the original barley grain (Townesley, 1979). Protein and fibre account for 20 % and 70 % of BSG dry matter respectively, while the starch content of BSG is insignificant (due to the absence of starchy endosperm). Due to its protein-rich composition, BSG has the potential to be utilised in a manner similar to whey protein, providing health benefits for consumers. BSG is also rich in phenolic compounds, particularly ferulic acid and *p*-coumaric acid (Bartolome *et al.*, 2002), along with oligosaccharides and polysaccharides (Mussatto *et al.*, 2006). Emerging evidence, with regard to the ability of dietary phenolic compounds to exhibit anti-carcinogenic, anti-inflammatory and antioxidant activities (Nagasaka *et al.*, 2007; Yang *et al.*, 2001), has led to significant interest in plant phenolic compounds particularly by the food industry, scientists and consumers.

To date, BSG has been widely used as an animal feed, particularly for cattle, to provide high amounts of both protein and fibre. BSG is an excellent feed ingredient for ruminants, providing all the essential amino acids when combined with inexpensive nitrogen sources such as urea (Huige, 1994). However, with the increased cost of disposal of the solid fraction, alternative uses are highly sought-after and it has been shown that BSG can be effectively integrated into ready-to-eat snacks to increase dietary fibre, crude protein and fat levels (Stojceska *et al.*, 2008). Other areas of successful research include the blending of BSG with flour for incorporation into cookies (Prentice *et al.*, 1978) and

the addition of BSG to dough to improve the dietary fibre content in bread (Stojceska & Ainsworth, 2008).

This review details existing evidence regarding BSG. A specific focus is placed on the potential bioactivities of phenolic compounds (particularly ferulic acid and *p*-coumaric acid) present in BSG, and the incorporation of BSG into foodstuffs, for both human and animal consumption.

1.2.3. Composition of BSG

Many studies have reported on the proximate composition of BSG, which contains protein, fat, cellulose, hemicelluloses and lignin (Table 1). As shown, there is good consistency with regard to the composition of BSG. However, variations can arise due to differences in barley variety, harvesting time, characteristics of hops added and brewery technology (Santos *et al.*, 2003). BSG predominantly consists of the husk-pericarp-seed coat layers that are rich in cellulose, non-cellulosic polysaccharides, lignin, protein and fat. This is reflected in the composition of BSG (Table 1), and thus BSG can be regarded as a lignocellulosic material (Mussatto *et al.*, 2006). In addition to the components detailed in Table 1, it has been shown that BSG is also a valuable source of vitamins, minerals and amino acids, particularly for animal feeding. The vitamins present in BSG are biotin, folic acid, niacin, choline, riboflavin and thiamine, pantothenic acid, pyroxidine (Huige, 1994). BSG is also reported to contain minerals such as calcium, copper, iron, manganese, potassium and sodium (Huige, 1994; Pomeranz *et al.*, 1976) and both essential (including lysine, histidine, methionine, phenylalanine, tryptophan) and non-essential (including alanine, serine, glycine, proline) amino acids (Huige, 1994). When combined with inexpensive nitrogen sources, such as urea, BSG can provide all the essential amino acids to ruminant animals (Huige, 1994).

Table 1: The proximate chemical composition of brewers' spent grain (BSG).

Study	Component (as % dry matter ^a)						
	Protein	Lipid/Oil	Ash	Cellulose	Hemicelluloses	Lignin	Starch
Beldman <i>et al.</i> , 1987	23.8	-	3.5	15.1	24.8	-	2.0
Valverde, 1994	24.0	6.0	-	17.0	39.0	4.0	-
Kanauchi <i>et al.</i> , 2001	24.0	10.6	2.4	25.4	21.8	11.9	-
Santos <i>et al.</i> , 2003	24.2	3.9	3.4	-	-	-	-
El-Shafey <i>et al.</i> , 2004	26.7	8.9	3.9	-	-	5.3	-
Mussatto & Roberto, 2005	15.3	-	4.6	16.8	28.4	27.8	-
Xiros <i>et al.</i> , 2008	14.2	13.3	3.3	12.0	40.2	11.5	-
Treimo <i>et al.</i> , 2009	23.4	-	-	-	-	12.6	7.8

^a Values expressed as % dry matter, which has been documented in two studies to be 20.4 % (Beldman *et al.*, 1987) and 20 % (El-Shafey *et al.*, 2004).

1.2.4. Phenolic component of BSG

1.2.4.1. Phenolics present in BSG

Phenolic acids, particularly hydroxycinnamic acids and hydroxybenzoic acids are secondary plant metabolites found extensively in plant foods. Phenolic acids are currently the focus of much attention due to their potential to act as antioxidant, anti-inflammatory and anti-carcinogenic compounds (Nagasaka *et al.*, 2007; Yang *et al.*, 2001).

As previously mentioned, BSG consists predominantly of the husk-pericarp-seed coat and is largely made up of cell walls. Since most of the phenolic compounds of the barley grain are contained in the husk (Mussatto *et al.*, 2006) and hydroxycinnamic acids accumulate in cell walls, BSG is a potentially valuable source of phenolic acids.

There is evidence to suggest that ferulic acid and *p*-coumaric acid (as shown in Figure 1) are present at relatively high concentrations in BSG (Bartolome *et al.*, 2002). Some of the existing literature regarding the presence of ferulic and *p*-coumaric acid is detailed in Table 2. Ferulic acid was found to be the most abundant hydroxycinnamic acid being present at concentrations ranging from 1860-1948 µg/g, while the *p*-coumaric levels ranged from 565-794 µg/g (Hernanz *et al.*, 2001). More recent evidence shows that BSG consists of 1.16 % mono and dimeric phenolic acids, with 53 % of the monomeric phenolic acids accounted for by ferulic acid. The vast majority of phenolic acids were also found to be in the bound form (Forssell *et al.*, 2008). It has been reported that following ferulic and *p*-coumaric acids, the next most abundant phenolic acids in BSG were found to be sinapic, caffeic and syringic acid (Szwajgier *et al.*, 2010). A summary of the phenolic acids present in BSG is given in Table 3.

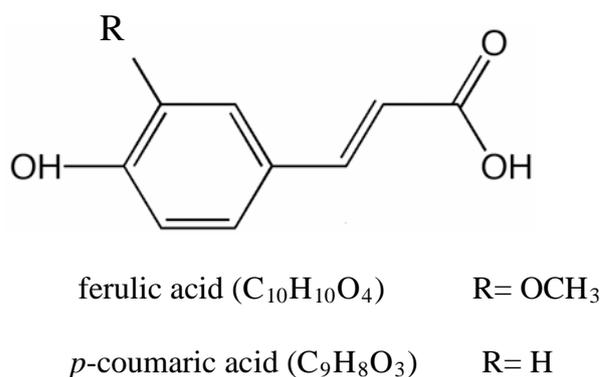


Figure 1: The general structure of hydroxycinnamic acid and the functional groups for ferulic and *p*-coumaric acids.

Table 2: The percentage (% dry weight) of bound phenolics, ferulic acid and *p*-coumaric acid present in brewers' spent grain (BSG).

Study	Percentage (as % dry matter)		
	Bound Phenolics	ferulic acid	<i>p</i> -coumaric acid
Bartolome <i>et al.</i> , 2002	-	~0.2	~0.1
Mandalari <i>et al.</i> , 2005	-	0.6	0.3
Athanasios <i>et al.</i> , 2007	-	0.2-0.3	0.1-0.2
Forsell <i>et al.</i> , 2008	-	0.5	0.5
Treimo <i>et al.</i> , 2009	0.7	-	-
Robertson <i>et al.</i> , 2010	0.8	-	-
Szwajgier <i>et al.</i> , 2010	-	0.3	0.1

Table 3: The most abundant phenolic acids present in brewers' spent grain, modified from Sz wajgier *et al.*, (2010)

Phenolic acid	Free acid concentration
	(mg/100g dry matter)
	Mean ± SE
Ferulic acid	336.3 ± 16.0
<i>p</i> -Coumaric acid	64.4 ± 4.6
Sinapic acid	42.0 ± 1.1
Caffeic acid	9.9 ± 0.7
Syringic acid	6.5 ± 0.1
4-OH-Benzoic acid	1.2 ± 0.6
Chlorogenic acid	0.6 ± 0.2
Protocatechuic acid	0.5 ± 0.1

1.2.4.2. Extraction of phenolic acids from BSG

Numerous studies have been conducted to extract phenolic acids from BSG. Novel techniques for extraction, such as a rapid microwave-assisted derivatization process have been investigated (Athanasios *et al.*, 2007). However, the majority of approaches use the basis of either acid hydrolysis or saponification (with 1 - 4 M NaOH) and liquid-liquid or liquid-solid extraction. A review of methods of extracting, separating and detecting phenolic acids in natural plant foods showed that the most frequently used methods involve acid hydrolysis and saponification (Stalikas, 2007). Extraction usually entails the use of solvents such as methanol and ethyl acetate. Thin layer chromatography (TLC) is extensively used for detecting phenolic acids due to its high sample throughput. However, using high performance liquid chromatography (HPLC) gives a greater degree of separation of compounds and is highly reproducible where quantification is possible. Therefore, reverse-phase (RP-) HPLC is predominantly used, but HPLC coupled with ultraviolet (UV) or diode array detection (DAD) is also an option (Zgórk & Kawka, 2001). A review looking at the extraction and quantification of phenolics in foods also reported that methanolic extraction and alkaline hydrolysis are commonly used for phenolic acid extraction, while a sequential alkaline hydrolysis releases bound phenolics (Nacz & Shahidi, 2004). A new method has recently been developed and validated, for the release of phenolic acids (both free and bound) from cereals including barley. This method uses solid-phase extraction (SPE) coupled with HPLC-DAD analysis and is simple, inexpensive and gives good recoveries and precision (Irakli *et al.*, 2012). Recently published results show that exogenous ferulic acid esterase (FAE) produced by the probiotic organism *Lactobacillus acidophilus* K1 can successfully release the free phenolics from BSG (Szwajgier, 2011). In 2005, a study looking at the hydroxycinnamate content of BSG fractions utilized saponification with 4 M NaOH. The supernatants were then neutralised and extracted with ethyl

acetate, dried and re-suspended in MetOH:H₂O (Mandalari *et al.*, 2005). More recent research also used this method, giving comparable results (Robertson *et al.*, 2010). Using a LUNA C18 RP-HPLC column, both studies found that ferulic acid was the phenolic acid in greatest abundance in BSG, with coumaric acid being second highest. Saponification (involving the treatment of samples with 1 - 4 M NaOH solution) has been widely used to extract hydroxycinnamic acids from BSG (Faulds *et al.*, 2004; Bartolome *et al.*, 2002; Hernanz *et al.*, 2001).

Extraction methods similar to those used for BSG, have also been utilised with other materials such as wheat bran extracts (Kim *et al.*, 2006) and apple waste extracts (McCann *et al.*, 2007).

1.2.4.3. Potential health benefits of phenolic component of BSG – in vitro evidence

As previously mentioned, ferulic and *p*-coumaric acids are the phenolic acids found in the highest concentrations in BSG (Table 3). Much research has been conducted looking at the *in vitro* antioxidant activity of hydroxycinnamic acids, particularly ferulic and *p*-coumaric acids, however evidence regarding *in vivo* antioxidant effects are limited to date. A commonly used method for quantification of antioxidant activity is the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, which measures the ability of the test compound to scavenge the DPPH radical. Chen & Ho (1997) and others have shown the antioxidant potential of ferulic acid using the DPPH and Rancimat methods, but ferulic acid was a less potent antioxidant than caffeic acid and α -tocopherol (Chen & Ho, 1997; Brand-Williams *et al.*, 1995). Caffeic acid has been shown to act as an antioxidant *in vitro* and scavenged radicals including DPPH and the superoxide anion (Gulcin, 2006). It has also been shown, using the DPPH assay, that a number of hydroxycinnamic acids act as antioxidants, scavenging DPPH in the order caffeic acid > sinapic acid = ferulic acid > ferulates > *p*-coumaric acid (Kikuzaki *et al.*, 2002). Similarly, but using an

alternative laboratory method, a study investigating the phenolic compounds in wheat bran extract and their antioxidant activity again found that ferulic acid was one of the strongest antioxidants using the β -Carotene Linoleic Acid Model System (β -CLAMS). The β -CLAMs assay is based on the principle that at high temperature the oxidation of linoleic acid produces peroxides that decolourise β -carotene. The wheat bran extracts with highest ferulic acid concentrations (following alkaline hydrolysis) also exhibited higher antioxidant activity (Kim *et al.*, 2006). Ferulic acid and caffeic acid have been reported to have excellent antioxidant potential at low concentrations, with the ability to scavenge a range of free radicals. Both phenolic acids scavenge the reactive oxygen species (ROS) and reactive nitrogen species (RNS), with concentration dependent scavenging of nitric oxide (NO), superoxide and 2,20-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid radical (ABTS⁺). In line with previously discussed evidence, caffeic acid was a stronger scavenger of the DPPH radical, but ferulic acid was better at scavenging ABTS⁺ and NO (Maurya & Devasagayam, 2010). In a recent study on beers, a direct correlation was found between ferric reducing antioxidant power (FRAP) and a number of phenolic acids including ferulic, *p*-coumaric, caffeic, sinapic and vanillic acids (Piazzon *et al.*, 2010). A second study also showed that some of the phenolic acids present in beer correlate with the antioxidant activity measured by DPPH radical and superoxide anion scavenging, metal chelation and reducing power; these include syringic and caffeic acids (Zhao *et al.*, 2010). Since ferulic acid is so well recognised as an antioxidant, it is approved for use as a food additive in some countries to prevent oxidation (Itagaki *et al.*, 2009; Graf, 1992). In addition, it is important to note that while phenolic compounds can have an *in vitro* antioxidant effect, they have also been shown to act as pro-oxidants under certain conditions, thus inducing oxidative stress. Recent literature suggests that at low concentrations, many phenolics exhibit pro-oxidant behaviour, whereas the synthetic antioxidants, including α -tocopherol, do not

(Fukumoto & Mazza, 2000). For caffeic acid and ferulic acid to act as pro-oxidants, higher concentrations ($> 5 \mu\text{M}$) are required (Maurya & Devasagayam, 2010). It has also been found using the comet assay that at high concentrations *p*-coumaric acid enhanced DNA breakage induced by hydrogen peroxide. This may be due to the production of ROS by *p*-coumaric acid as a result of its pro-oxidant activity (Ferguson *et al.*, 2005). It has been suggested that this pro-oxidant effect is related to the presence of metal ions in the body (for example due to tissue injury releasing iron and copper) and is of relevance for the bioactivity of phenolic compounds *in vivo* (Morton *et al.*, 2000). It is clear from a small number of *in vivo* studies that the hydroxycinnamic acids have antioxidant properties. Such studies are essential to understand the biological role of these phenolic acids (Shahidi & Chandrasekara, 2010).

In addition to their *in vitro* antioxidant activity, there is increasing evidence to suggest that phenolic acids can have an anti-carcinogenic effect. Caffeic acid ($50 \mu\text{M}$) exhibits an anti-proliferative effect on several cancer cells including mammary gland, adenocarcinoma, lymphoblastic leukaemia (Gomes *et al.*, 2003) and cervical cancer cell lines as assessed using the MTT assay (Chang *et al.*, 2010; Gomes *et al.*, 2003). The cyclooxygenase-isoform 2 (COX-2) assay has been used for determination of the anti-cancer potential of these compounds. Over expression of COX-2 increases the conversion of arachidonic acid to prostaglandins, which are important mediators of inflammation, and are associated with cancer. Phenolic acids including caffeic acid (Kang *et al.*, 2009) and vanillic acid (Kim *et al.*, 2011), and polyphenols including epigallocatechin-3-gallate (Hussain *et al.*, 2005) and quercetin (Garcia-Mediavilla *et al.*, 2007), have been shown to inhibit the expression of COX-2, possibly reducing cancer risk. Apoptosis in cancer cell lines is also an indicator of anti-carcinogenic potential and can be assessed by a number of methods including DNA fragmentation and the Hoechst staining assay. Cinnamic acid derivatives induced apoptosis in human leukaemia

(HL60) and colon cancer (SW480) cell lines, as measured by the aforementioned apoptosis methods (Akao *et al.*, 2003). Additionally, the anti-apoptotic effect of phenolic compounds including ferulic acid and caffeic acid on human peripheral blood mononuclear cells (PBMCs) was investigated (Khanduja *et al.*, 2006). Caffeic acid inhibited externalisation of phosphatidyl serine (PS), which indicates the pre-apoptotic stages, and hence it was concluded to have an anti-apoptotic effect. DNA fragmentation was analysed using an apoptotic DNA ladder kit. It was shown that pre-treating cells with caffeic acid, ferulic acid or ellagic acid before exposure to H₂O₂ inhibited DNA fragmentation. Recently published data adds to the evidence available in the area, by measuring the ability of phenolic compounds to modulate nuclear factor kappa B (NF-κB) activity. In the inflammatory process, NF-κB is a transcription factor, whose increased activation has been reported in several human cancers (Escárcega *et al.*, 2007). Free phenolic acids which can be found in cereal grains (including ferulic, caffeic, sinapic and *p*-coumaric acids) significantly modulate NF-κB activity in U9373κB-LUC cells, with a desired level of modulation being achieved by the synergistic action of phenolic acids and other phenolic compounds (Hole *et al.*, 2012). Animal studies have also been carried out to determine the anti-carcinogenic potential of phenolic acids. An animal study to establish the effect of curcumin, chlorogenic acid, ferulic acid and caffeic acid on tumor promotion in the skin of mice showed that chlorogenic, ferulic and caffeic acid prevented the number of 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced tumours per mouse by 60, 28 and 35 %, respectively (Huang *et al.*, 1988). Results of a later animal study suggest that ferulic acid not only inhibits the growth of aberrant crypt foci (ACF) in the colon, but also prevents the conversion of pre-neoplasia to malignant neoplasia (Kawabata *et al.*, 2000). A recently published review on plant phenolics reported that natural phenolics, including those present in tea and fruit polyphenols, play an antagonistic role in all

stages of cancer development and that further study on these compounds will provide information regarding their possible future pharmaceutical use (Dai & Mumper, 2010).

Cytokines are small cell signalling molecules involved in the inflammatory response, these include interleukins and interferons (for example interferon-gamma (IFN- γ)). The ability of a compound to alter the production of a stimulated cytokine or NO indicates the compound's potential to act as an immune-modulator. Murakami *et al.* (2002) investigated the effect of both ferulic acid (from rice bran) and FA15 (a derivative of ferulic acid) on NOS, COX-2 and tumor necrosis factor- α (TNF- α) in the RAW264.7 murine macrophage cell line. Unlike the ferulic acid isolated from rice bran, the synthesised FA15 derivative was found to inhibit the release of TNF- α and reduce the protein expression of both NOS and COX-2 (Murakami *et al.*, 2002). Ferulic acid has also been shown to inhibit macrophage inflammatory protein-2 (MIP-2) and TNF- α production, induced by lipopolysaccharide (LPS) in a macrophage cell line. The effect, although dose-dependent, was very weak compared to the effect of dexamethasone (a well known inhibitor of interleukins) (Sakai *et al.*, 1997). In Japanese Oriental medicines, *Cimicifuga heracleifolia* (CH) is often used as an anti-inflammatory drug. Ferulic acid has been shown to be among the main phenolic acids in CH (He *et al.*, 2006). Sakai *et al.* (1999) showed that ferulic acid and isoferulic acid could reduce MIP-2 production in a dose-dependent manner in RAW264.7 cells. It was suggested that ferulic acid and isoferulic acid are responsible, at least in part, for the anti-inflammatory properties of the CH drug (Sakai *et al.*, 1999). Recently published data has shown that ferulic acid and *p*-coumaric inhibited LPS induced NO production and inducible NOS (iNOS) in macrophages (Kim *et al.*, 2012). This supports earlier evidence suggesting that these phenolic acids can act as anti-inflammatory agents, by reducing TNF- α induced interleukin-6 (IL-6) production in adipocytes. Quercetin, *p*-

coumaric acid and resveratrol showed greatest inhibition of IL-6 production (Yen *et al.*, 2011).

Oxidised low density lipoprotein (LDL) is a well recognised risk marker of cardiovascular disease which is principally caused by atherosclerosis (Yoshida & Kisugi, 2010). Evidence exists for the effect of hydroxycinnamic acids on the inhibition of low density lipoprotein oxidation. Nardini *et al.* (1995) demonstrated the antioxidant effect of hydroxycinnamic acid derivatives such as caffeic, ferulic and *p*-coumaric acids on LDL oxidation *in vitro*, with the use of the copper ion Cu^{2+} as a catalyst. At a concentration of 100 μM , all phenolic acids except *p*-coumaric acid inhibited LDL oxidation; at 20 μM , ferulic acid inhibited $\sim 92\%$ of copper-catalysed human LDL oxidation; at 5 μM only caffeic acid strongly inhibited the oxidation of LDL (Nardini *et al.*, 1995). A second study using similar methodology also found that both ferulic and *p*-coumaric acid showed a dose-dependent inhibition of human LDL oxidation *in vitro* when tested at 5, 10 and 20 μM (Meyer *et al.*, 1998). In agreement with these data, results of a later study showed sinapic, ferulic and *p*-coumaric acids inhibited LDL oxidation (Andreasen *et al.*, 2001). Other compounds that have shown the ability to reduce copper-induced LDL oxidation include catechin (Chen *et al.*, 2011).

Our research group is the first, to our knowledge, to look specifically at phenolic extracts from BSG. Four extracts from pale BSG (P1, P2, P3, P4) and four extracts from black BSG (where the grain is roasted to 200 °C before brewing, B1, B2, B3, B4) were analysed. Each extract results from a different step in the extraction process and hence contains different phenolic acid composition. Extract 1 (P1, B1) contains free phenolics, extracts 2 (P2, B2) contains bound phenolics, extract 3 (P3, B3) contains the remainder of bound phenolics and extract 4 (P4, B4) contains phenolics extracted with 110 mM NaOH (McCarthy *et al.*, 2012). The ability of the phenolic extracts to protect against oxidant-induced DNA damage was determined using the comet assay. In the U937 cell

line, oxidative DNA damage was induced by a range of oxidants; hydrogen peroxide (H_2O_2), 3-morpholinosydnonimine hydrochloride (SIN-1), 4-nitroquinoline oxide (4-NQO) and *tert*-butylhydroperoxide (*t*-BOOH). Table 4 shows the ability of the extracts to protect the cells against DNA damage. There was no protection against DNA damage induced by either 4-NQO or *t*-BOOH. Ferulic acid and the black BSG extracts significantly reduce the DNA damage induced by H_2O_2 , while P2, B2, B3, B4 significantly reduce the percent tail DNA induced by SIN-1 (McCarthy *et al.*, 2012). The four oxidants used have different mechanisms of action; damage induced by both H_2O_2 and SIN-1 involve the Fenton reaction and is an iron dependent reaction, 4-NQO mimics the action of UV and Cu^{2+} plays an essential role, while iron does not (Yamamoto *et al.*, 1993), *t*-BOOH causes lipid peroxidation and acts in a Ca^{2+} -dependent manner and iron plays less of a role than in H_2O_2 -induced damage (Kruszewski *et al.*, 2008). Therefore, BSG phenolic extracts may provide protection against oxidant-induced DNA damage by Fe chelation (McCarthy *et al.*, 2012).

In summary, there is increasing evidence to suggest that phenolic acids, including those found at highest concentrations in BSG, can confer potential health benefits including anti-inflammatory, antioxidant, anti-carcinogenic and anti-atherogenic effects. Recent data suggest that BSG has antioxidant potential and therefore further research on the phenolic compounds extracted from BSG is warranted.

1.2.5. Incorporation of BSG into feed/foodstuffs

1.2.5.1. Animal food

As previously mentioned, BSG contains approximately 20 and 70 % protein and fibre, respectively and it is due to this favourable chemical composition that it has great potential for use as a raw material/food ingredient (Mussatto *et al.*, 2006). BSG is an ingredient of significant importance for ruminants. When administered with low-cost

Table 4: DNA damage in U937 cells following 24 h incubation with 0.5 % v/v pale (P1 - P4) or black (B1 - B4) BSG phenolic extracts or 0.1 µg/mL ferulic acid (adapted from McCarthy *et al.*, 2012).

Sample ^a	DNA damage (% tail DNA) Oxidant – H ₂ O ₂	DNA damage (% tail DNA) Oxidant – SIN-1	DNA damage (% tail DNA) Oxidant – 4-NQO	DNA damage (% tail DNA) Oxidant – <i>t</i> -BOOH
Control	3.2 ± 0.5 ^b	4.3 ± 0.4 ^b	3.1 ± 0.3 ^b	4.7 ± 0.6 ^b
Oxidant control	40.3 ± 0.3 ^b	40.0 ± 3.9 ^b	39.5 ± 1.1 ^b	15.5 ± 0.8 ^b
P1	30.3 ± 7.3 ^b	32.8 ± 4.9 ^b	39.0 ± 6.8 ^b	14.5 ± 0.8 ^b
P2	29.0 ± 6.0 ^b	22.7 ± 1.9 ^{b*}	40.7 ± 4.3 ^b	22.6 ± 3.0 ^b
P3	39.9 ± 3.9 ^b	33.8 ± 2.9 ^b	37.3 ± 3.2 ^b	22.1 ± 3.1 ^b
P4	23.1 ± 3.2 ^b	32.2 ± 6.1 ^b	37.8 ± 3.6 ^b	17.4 ± 2.6 ^b
B1	25.8 ± 7.5 ^{b*}	33.6 ± 2.9 ^b	36.1 ± 5.7 ^b	13.6 ± 0.9 ^b
B2	9.6 ± 1.1 ^{b*}	6.9 ± 1.0 ^{b*}	31.9 ± 3.2 ^b	18.5 ± 3.3 ^b
B3	16.0 ± 2.0 ^{b*}	18.1 ± 0.3 ^{b*}	33.4 ± 2.4 ^b	16.6 ± 1.8 ^b
B4	14.0 ± 3.8 ^{b*}	24.1 ± 2.2 ^{b*}	33.7 ± 6.8 ^b	14.0 ± 2.7 ^b
Ferulic acid	17.1 ± 0.8 ^{b*}	30.8 ± 3.0 ^b	32.6 ± 5.2 ^b	11.0 ± 1.9 ^b

^a P1 - P4 represent the four pale BSG phenolic extracts, B1 - B4 represent the black BSG phenolic extracts. ^b Data represents the mean ± SE of at least three independent experiments. * Denotes a significant difference in DNA damage (P < 0.05), relative to oxidant control. Statistical analysis by ANOVA followed by Dunnett's test.

nitrogen sources such as urea, BSG can supply all the essential amino acids to ruminants. The effect of BSG on milk yield and composition, and the blood components of dairy cattle has also been studied (Belibasakia & Tsirgogianni, 1996). The cattle received a diet consisting of ground maize, maize silage, soya bean meal and wheat bran, with the latter three being substituted with wet brewer's grain in the treatment group. The study showed the treatment group had an increased milk yield, milkfat and milk total solids content. Blood components such as glucose, cholesterol, sodium, triglycerides were not affected. While the main outlet for BSG is currently as a feedstuff for dairy cattle, research has also been conducted looking at the benefits of BSG for use as a feed for poultry and fish. The effect of replacing rice bran in a fish diet with 10 - 40 % brewery waste grains has been investigated (Kaur & Saxena, 2004). The brewery waste used contained 19 % crude protein, 18 - 20 % crude fibre and had a good amino acid profile. It was found that carp (oily freshwater fish) had better growth performance on diets containing brewery waste than the control group. The authors attributed this enhanced growth performance to the high-quality protein contained in the waste grains. A more recent study showed that biodegraded BSG contained cysteine, lysine and methionine in addition to 14 other amino acids (Essien & Udotong, 2008). Depending on the microbe used to degrade the BSG, different amino acid concentrations were found, with alanine consistently at highest concentrations. This composition was noted to be of particular importance for poultry as cysteine, lysine and methionine are the main amino acids required in poultry nutrition. For convenience, the possibility of producing dry BSG cakes suitable for long term storage was examined using membrane filter press technology (El-Shafey *et al.*, 2004). These dry cakes could be used as an animal feed at any time, or as a starting material for the production of other products using BSG.

In summary, evidence suggests that whole BSG, fed as part of a total mixed ration, has many nutritional benefits for a range of animals, particularly dairy cattle. This has resulted in the routine use of BSG as an animal feed for cattle.

1.2.5.2. Human food

In addition to its use as an animal feed, BSG has been incorporated into foodstuffs for human consumption. Given its low cost and high nutritional value, BSG makes an ideal ingredient for human foods such as biscuits and ready-to-eat snacks, particularly where there is a need to increase fibre content. In 1978, the possibility of preparing cookies with flour containing BSG at levels ranging from 5 - 60 % was examined (Prentice *et al.*, 1978). At 40 % BSG addition, the physical qualities of the cookies were sustained. This supplementation level gave a 74 % increase in nitrogen and increased crude fibre ten-fold. These results were supported by work published in 2002, where the authors looked at the effect of BSG (at levels of 5 - 25 %) on the fibre content and quality of cookies (Öztürk, 2002). As the level of addition of BSG increased, there was a significant increase in dietary fibre content. Another documented foodstuff suitable for the inclusion of BSG is ready-to-eat snacks (Stojceska *et al.*, 2008). BSG was added to the formulation mix (consisting of ingredients such as corn starch and wheat flour) at levels ranging from 10 to 30 %. The incorporation was successful, increasing dietary fibre and crude protein levels. Similarly, the addition of BSG into extruded snack food has been studied (Ainsworth *et al.*, 2007). The maize flour of the chick pea snacks was replaced with BSG at levels of 10, 20, 25 and 30 %. The parameters measured included the effect of BSG supplementation on texture, colour, moisture, fat, fibre, starch, protein, phenolic compounds and antioxidant capacity. With increasing levels of BSG addition, the percent protein, fat and fibre content increased, while starch decreased. It was suggested that foods fortified with BSG be considered as functional foods. In a

further study by Stojceska and Ainsworth (2008), BSG was incorporated at different levels (0 – 30 %) into wheat flour breads treated with 4 different enzymes and the bread quality was then evaluated. Similar to the previous study, it was found that the fibre content of the breads was significantly increased by BSG addition. The change in fat content was significantly linked to the addition of BSG. When addition of BSG is combined with the appropriate use of enzymes, the shelf life, texture and loaf volume can also be improved. Initially, it was thought that BSG was too granular for direct addition to food and that it would have to first be converted to flour before use. However, a study in 2009 demonstrated that BSG of various particle sizes could be effectively used in the production of frankfurters (Özvural *et al.*, 2009). The control frankfurter had the highest score for acceptability, but the other products also had high scores, with the score decreasing with increase in particle size and reduction in fat levels. The authors suggested that BSG be used to produce low-fat high-fibre meat products. In addition to particle size, there are a number of points for consideration with the incorporation of BSG into foodstuffs. Firstly, there are concerns about appearance. When moist, BSG is brown in colour, thus can only be effectively integrated into off-white products. Such foods include cookies and cakes. More importantly, it is imperative that the organoleptic properties of the foodstuff remain acceptable to consumers and are similar to current commercially available products. The study by Prentice *et al.* (1978), demonstrated that BSG addition at a level of 15 % was the upper limit for organoleptic acceptability. At this level, the organoleptic quality was lowered but still remained acceptable to consumer panels. Similarly, Stojceska *et al.* (2008) found that there was a limit of acceptability. At a level of addition of 30 %, physicochemical characteristics (such as texture, colour, and hardness) remained acceptable. However, the authors concluded that addition of BSG at 20 % level was optimal to maintain properties of commercially available snack foods. Where the

protein hydrolysates are to be extracted and incorporated into foodstuffs, there is concern over the bitter taste of some peptides, due to the hydrophobic amino acid content (Clemente, 2000).

1.2.6. Conclusion

The literature shows that phenolic compounds including ferulic, caffeic and *p*-coumaric acid can exert antioxidant, anti-cancer, anti-atherogenic and anti-inflammatory effects *in vitro*. Given that these phenolic acids are some of the major phenolics in BSG, it is expected that phenolic extracts from spent grain may also exhibit similar properties and have the potential to be developed for a range of bioactivities. BSG currently functions as an animal feed, having many nutritional benefits. While some attempts have been made to incorporate the bioactive components of BSG into foodstuffs, further research in this area is needed. Given the potential bioactive nature of the phenolic extracts from BSG, and the large amounts of BSG produced annually as a low value co-product, it is imperative that an alternative use be explored.

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1.3. Protein hydrolysates from agricultural crops—bioactivity and potential for functional food development.

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1.3.1. Abstract

There has been an unprecedented demand for inexpensive plant-derived protein hydrolysates in recent years, owing to their potential nutritional applications. This review examines existing evidence regarding protein hydrolysates from agricultural crops such as wheat, soy, rapeseed, sunflower and barley. The *in vitro* bioactivity of these protein hydrolysates, including antioxidant and anti-hypertensive capabilities are discussed. In addition to evidence regarding their potential to enhance human nutrition, the effect of the hydrolysates on the techno-functional properties of foods will be reviewed.

1.3.2. Introduction

Humans require a protein intake sufficient to maintain the body nitrogen balance and allow for desirable rates of deposition during growth and pregnancy. Ingestion of protein amounts greater than requirements results in the excess being metabolised and excreted. Conversely, in the case of inadequate dietary protein intake, the body utilises its own proteins as a source of nitrogen; therefore a regular and sufficient intake is essential. Protein performs a number of key functions in the body including the building and repair of tissues, cell signalling and the provision of energy (4 kcal/g protein). Proteins also perform enzymatic and structural functions.

Protein hydrolysates have been defined as “mixtures of polypeptides, oligopeptides and amino acids that are manufactured from protein sources using partial hydrolysis” (Schaafsma, 2009). There has been growing interest in these preparations over the last two decades, with novel bioactive peptides continually being discovered, as it has been shown that short-chain peptides from hydrolysed proteins have a higher nutritive value and may

be utilised more efficiently than an equivalent mixture of free amino acids (Grimble *et al.*, 1986). Milk-based products are the source of the greatest number of bioactive peptides isolated to date. Other sources include meat, eggs and fish, in addition to plant sources such as soy and wheat (Hartmann & Meisel, 2007). Figure 1 illustrates the effect of bioactive peptides on major body systems. It has emerged that protein hydrolysates have many uses in human nutrition; ingredients in energy drinks, weight-control and sports nutrition products (Clemente, 2000), sources of nutrition for elderly and immuno-compromised patients (Nagodawithana *et al.*, 2010). Applications include the incorporation of protein hydrolysates into energy drinks, weight-control and sports nutrition products (Frokjaer, 1994). Clinical applications have also been suggested, for the treatment of Phenylketonuria (PKU), liver disease, Crohn's disease and ulcerative colitis (Clemente, 2000). Other functions of plant-derived protein hydrolysates have been discussed in detail elsewhere (Pasupuleti *et al.*, 2010). These include use as natural herbicides, particularly corn gluten meal and soy and wheat hydrolysates (Christians *et al.*, 1994) and as replacements for materials of bovine origin in fermentation media, to reduce risk of Bovine Spongiform Encephalopathy (BSE) contamination (Ranganathan 2010).

In recent years there has been an unprecedented demand by both consumers and industry, for inexpensive plant-derived proteins and bioactive peptides for human consumption. Additionally, alternative uses for co-products of the plant processing industry are highly sought. Such co-products include brewers' spent grain (BSG), wheat bran and okara (a soybean by-product of tofu production), which are excellent sources of both protein and fibre (Mandalari *et al.*, 2005; O'Toole, 1999).

The present review focuses on the *in vitro* bioactivity of protein hydrolysates from a range of agricultural crops, and their potential for inclusion into functional foods.

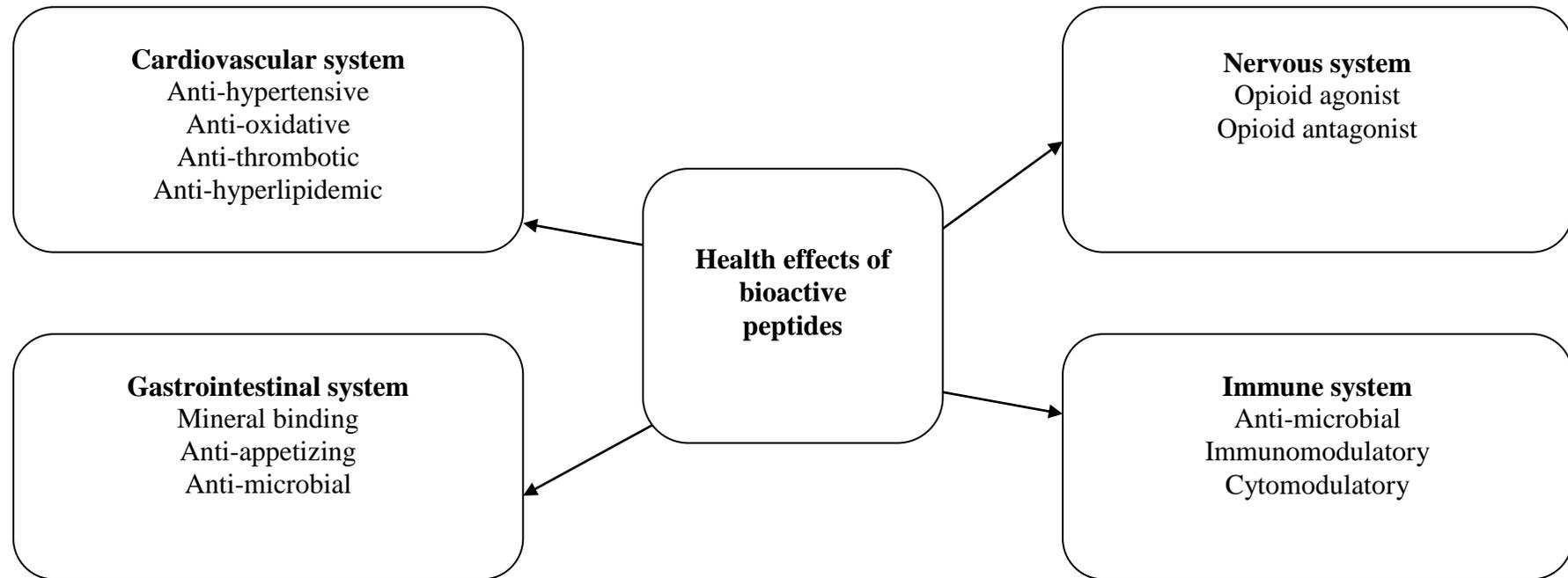


Figure 1: Physiological effects of food derived bioactive proteins on major body systems.

1.3.3. Preparation of protein hydrolysates

1.3.3.1. Protein hydrolysis

Hydrolysis of proteins involves the cleavage of peptide bonds to give peptides of varying sizes and amino acid composition. There are a number of types of hydrolysis; enzymatic, acid or alkali hydrolysis. Chemical hydrolysis is difficult to control and reduces the nutritional quality of products (Celus *et al.*, 2007), destroying L-form amino acids and producing toxic substances such as lysino-alanine (Lahl & Grindstaff, 1989). Enzymatic hydrolysis works without destructing amino acids and by avoiding the extreme temperatures and pH levels required for chemical hydrolysis, the nutritional properties of the protein hydrolysates remain largely unaffected (Celus *et al.*, 2007). Production of protein hydrolysates in the food industry involves the use of digestive proteolytic enzymes from animals including chymotrypsin, trypsin and pepsin, or food grade enzymes obtained from plants and microorganisms which are regarded safe for human nutrition. Following protein hydrolysis, fractions can be categorised according to two characteristics. The first category consists of fractions with a high amino acid content. The second category consists of bioactive peptides with an amino acid sequence which is inactive in the intact protein molecule but becomes active in the hydrolysate following exposure to digestive and/or proteolytic enzymes (Schaafsma, 2009).

1.3.3.2. Post-hydrolysis treatment

Following hydrolysis, the 'crude hydrolysates' may undergo further processing. Commonly used post-hydrolysis processes include heat inactivation, ultrafiltration, hydrolysis by exoproteases and treatment with specific enzymes. Table 1 details the main post-hydrolysis processes and the function of each of these processes. Control of the molecular size of protein hydrolysates is an essential step in the development of protein hydrolysates for

Table 1. The main processes used following hydrolysis of protein (post-hydrolysis processes).

Process	Function
Heat inactivation	Inactivation of proteolytic enzymes
Ultrafiltration	Removal of high molecular weight proteins and peptides
Use of specific enzymes	Reduce content of specific amino acids
Hydrolysis by exoproteases	Hydrolysis, reduction of bitterness
Activated carbon	Reduction of bitterness
Absorption chromatography	Reduce content of aromatic amino acids

dietary use. Removal of high molecular weight proteins and peptides is primarily carried out using ultrafiltration. Protein hydrolysates can have a bitter taste and the elimination or reduction of this bitterness is essential to make the hydrolysates acceptable to consumers. The bitterness of protein hydrolysates is attributable to their hydrophobic amino acid content (Schaafsma, 2009) and the release of these amino acids by exoproteases can reduce bitterness (Pedersen, 1994). Post-hydrolysis processes can also be used to produce hydrolysates for the treatment of clinical conditions. For example, the use of phenylalanine ammonia lyase enzyme can reduce the phenylalanine content in protein hydrolysates, producing a hydrolysate suitable for patients with phenylketonuria, a disorder of phenylalanine metabolism (Clemente, 2000).

1.3.4. Bioactivity of protein hydrolysates

1.3.4.1. Antioxidant

Protein hydrolysates from agricultural crops including soy, rapeseed, wheat, sunflower and barley have been investigated for their antioxidant potential. In 1980, it was reported that following proteolysis, soy protein hydrolysates showed antioxidant potential, as measured by the thiobarbituric (TBA) assay, which is a measure of lipid peroxidation. It was suggested that the release of bound antioxidant phenolics or copper chelating agents was responsible for the observed antioxidant activity (Yee *et al.*, 1980). Using similar methodology, Peña-Ramos and Xiong (2002) provided comparable results. Soy protein hydrolysates prepared with Flavourzyme or chymotrypsin had antioxidant potential greater than unhydrolysed soy protein isolate. However, it was found that extensive degradation using enzymes such as papain unfavourably altered the antioxidant activity (Yee *et al.*, 1980). A further study measured the ability of these soy protein hydrolysates, prepared with Flavourzyme or chymotrypsin, to inhibit lipid oxidation in cooked pork patties. However, in contrast to the initial study, the soy protein hydrolysates did not affect lipid

oxidation by the TBA assay, but reduced conjugated diene (CD, a marker of free radicals) formation in stored pork patties (Peña-Ramos & Xiong, 2003). CD is a secondary product of lipid oxidation. Later studies have also reported the antioxidant potential of soy protein hydrolysates (Roblet *et al.*, 2012; Xin *et al.*, 2011; Moure *et al.*, 2006; Fan *et al.*, 2005). Soy protein hydrolysates post-treated with ultrafiltration, resulting in low molecular weight fractions (< 10 kDa) have shown greatest antioxidant potential (Moure *et al.*, 2006).

Protein hydrolysates from a range of other agricultural crops have been less extensively studied in comparison to soy hydrolysates. Rapeseed protein hydrolysates exhibited a dose-dependent inhibition of lipid peroxidation by a speculated proton donation mechanism (Zhang *et al.*, 2008). Later studies supported these findings, with rapeseed hydrolysates showing the ability to act as reducing agents and scavenge hydroxyl radical and superoxide anion (Pan *et al.*, 2011; Xue *et al.*, 2009). By employing the post-hydrolysis process of affinity chromatography, copper chelating peptides were isolated from sunflower protein hydrolysates. The ability to chelate copper increased mineral bioavailability and exerted antioxidant effects (Megias *et al.*, 2008; Megias *et al.*, 2007). Protein hydrolysates isolated from wheat germ also possess radical scavenging abilities, with an antioxidant activity close to that of the well known antioxidant α -tocopherol. Interestingly, these hydrolysates had low molecular weight < 1500 Da (Zhu *et al.*, 2006). Similarly, enzymatic hydrolysates of buckwheat showed excellent antioxidant potential, scavenging DPPH radical, inhibiting linoleic acid peroxidation and possessing reducing power (Tang *et al.*, 2009).

Protein hydrolysates isolated from co-products of the plant processing industry have also been investigated for antioxidant activity. While BSG protein isolate and associated hydrolysates do not possess antioxidant potential (McCarthy *et al.*, 2013), okara hydrolysates protect against oxidation of linoleic acid (Yokomizo *et al.*, 2002). Furthermore, it has been shown that fermentation of okara using *Bacillus subtilis* B2 can

greatly improve its antioxidant activity, thus adding value to this co-product (Zhu *et al.*, 2008).

1.3.4.2. Anti-hypertensive

Normal blood pressure is in the range of 100 - 140 mmHg (systolic) and 60 - 90 mmHg (diastolic). Values greater than 140 mmHg (systolic) and 90 mmHg (diastolic) are classified as hypertension or high blood pressure. In hypertension, the blood pressure in the arteries is elevated and the heart has to work hard to pump blood around the body. According to the World Health Organisation (2009), high blood pressure is particularly relevant in middle income European countries and African countries. A high percentage of stroke (51 %) and ischaemic heart disease (45 %) deaths worldwide are attributable to high systolic blood pressure. Dietary and lifestyle changes, including a reduction in salt intake and an increase in physical activity levels, can positively influence blood pressure. However, in cases where such changes are ineffective or insufficient, drug treatments may be prescribed. Angiotensin-converting enzyme (ACE) inhibitors are an example of a drug treatment to control blood pressure. ACE reduces the conversion of angiotensin-1 (vasodilatory) to angiotensin-2 (vasoconstrictory) resulting in a reduced blood pressure. Hence, there is great interest in novel compounds that can inhibit ACE. In 2000, wheat germ hydrolysate and its dominant peptide significantly reduced mean arterial pressure (MAP) in spontaneously hypertensive rats. It was shown that the dominant bioactive peptide could be metabolised by an aminopeptidase to form an ACE inhibitory metabolite, indicating potential blood pressure lowering effects of the metabolite after absorption (Matsui *et al.*, 2000). Similarly, a buckwheat protein hydrolysate was found to reduce systolic blood pressure in spontaneously hypertensive rats and also inhibit ACE, particularly when hydrolysis was carried out with pepsin followed by chymotrypsin and trypsin (Li *et al.*, 2002). More recently, it was found that ultrasonic pre-treatment promotes

the release of ACE inhibitory peptides during enzymatic proteolysis of wheat germ (Jia *et al.*, 2010). van der Ven *et al.* (2002) described the processing conditions necessary to produce hydrolysates with maximal ACE inhibitory activity. It was suggested that the ACE inhibitory activity of protein hydrolysates is due to the synergistic action of the different peptides present, thus the isolation of peptides is not justified and optimising the entire peptide composition is essential. Response surface modelling (which comprises a body of methods to explore optimum operating conditions through experimental methods (Lenth, 2009)) is effective in the optimisation of a number of parameters simultaneously to produce a hydrolysate with maximum ACE inhibitory activity. Similar to the results for antioxidant activity of protein hydrolysates, it was found that ACE inhibitory activity of soy protein hydrolysates increased with decreasing molecular weight of peptides, hence ACE inhibitory peptides have low molecular weight (Wu & Ding, 2002). This study also focused on an important consideration for the formulation of anti-hypertensive functional foods, that is the digestibility of the protein hydrolysates. Following *in vitro* gastric digestion, which simulates conditions in the human stomach, of the soy protein hydrolysates, the ACE inhibitory activity was retained. Stability during processing is another key factor and hydrolysates were shown to have sufficient stability to various heat (20 - 100 °C) and pH (pH 2- 10) treatments. These findings were supported by a study published in 2006, where it was also reported that soy protein hydrolysates possessed ACE inhibitory activity that was unaffected by *in vitro* gastrointestinal proteases (Chaing *et al.*, 2006). Protein hydrolysates from a range of other crops including potato (Pihlanto *et al.*, 2008), corn (Kim *et al.*, 2004; Suh *et al.*, 1999), spinach (Yang *et al.*, 2003), sunflower (Megias *et al.*, 2004; Megias *et al.*, 2009), peanut (Jamdar *et al.*, 2010) and rapeseed (Yoshie-Stark *et al.*, 2006) have exhibited high ACE inhibitory activities. Rice-bran, an under-utilized co-product of rice milling also has the potential to inhibit ACE activity with high molecular weight hydrolysates (10 - 50 and > 50 kDa) resulting in at least 50 % inhibition (Hull *et al.*, 2011).

In addition to ACE inhibition, there are a number of other potential mechanisms of inhibiting hypertension. These include activation of endothelial nitric oxide synthase (NOS), reduction of Ca^{2+} in vascular smooth muscle cells (VSMC) and rennin inhibition (Chen *et al.*, 2009). The ability of a compound to induce nitric oxide (NO) production via NOS and increase endothelial cell Ca^{2+} concentration contributes to vasodilation and reduced blood pressure (Chen *et al.*, 2009). Studies utilizing these mechanisms have also been carried out; for example soy protein isolate and hydrolysates have been shown to increase NO release in human aortic endothelial cells (HaoEC) (Ringseis *et al.*, 2005).

1.3.4.3. Cardiovascular disease

Cardiovascular diseases (CVD) are the primary cause of death globally, representing 30 % of all global deaths in 2008 (World Health Organisation, 2011). Cardiovascular diseases are diseases of the heart and blood vessels and include coronary heart disease, cerebrovascular disease and peripheral arterial disease (World Health Organisation, 2011). An unhealthy diet and physical inactivity are among the two main risk factors for CVD, resulting in raised blood pressure, blood lipids and blood glucose, overweight and obesity, which are classed as intermediate risk factors for CVD (American Heart Association, 2009). The consumption of vegetable protein has been associated with a lower risk of coronary heart disease, in comparison to consumption of animal protein (Clifton, 2011; Terpstra *et al.*, 1983; Hilleboe, 1957). This observed effect may be attributed to decreases in serum cholesterol levels (Manson *et al.*, 1992). For more than 100 years, animal studies have shown the cholesterol lowering effect of soy protein compared with animal protein (Ignatowsky, 1908). Reduced intestinal cholesterol absorption and increased faecal bile acid excretion, reduced levels of hepatic lipogenic enzymes such as glucose-6-phosphate dehydrogenase (G6PDH) and stimulation of adipopectin (a cytokine involved in adipocyte differentiation) and insulin sensitivity are all possible mechanisms for the lipid lowering

effect of soy protein (Velasquez & Bhatena, 2007). A meta-analysis of 38 studies indicated that soy protein consumption significantly decreased serum cholesterol, low density lipoprotein (LDL) cholesterol and triglyceride concentrations; there was also an increasing trend in high density lipoprotein (HDL) cholesterol concentrations (Anderson *et al.*, 1995). An animal study carried out on genetically obese mice and dietary obese rats measured the effect of soy protein isolate and hydrolysate and on the rate of body fat disappearance. Feeding with either soy isolate or hydrolysate resulted in a significantly reduced body-fat content and plasma glucose levels in comparison with control, casein fed rodents. A decrease in the plasma total cholesterol level was also observed (Aoyama *et al.*, 2000). Soy hydrolysates have also demonstrated effects such as decreased cholesterol absorption both *in vitro* and in rats (Nagaoka *et al.*, 1999), anti-adipogenic *in vitro* (Tsou *et al.*, 2010), reduced fat mass and serum lipid in rats (Park *et al.*, 2010). Sunflower hydrolysates produced using alcalase or pepsin inhibited the incorporation of cholesterol into bile salts micellar suspensions (Megias *et al.*, 2009b). As mentioned previously, *in vitro* digestion did not decrease ACE inhibitory bioactivity of soy protein hydrolysates. It has also been shown that digestion of sunflower protein hydrolysates with simulated intestinal fluids produces new peptides that inhibit cholesterol incorporation into micellar suspensions (Megias *et al.*, 2009). Protein hydrolysates from crop processing co-products have been less extensively studied, but rice bran hydrolysates have demonstrated hypocholesterolemic activity, by reducing total cholesterol and increasing HDL cholesterol in male Wistar rats (Revilla *et al.*, 2009).

1.3.4.4. Exercise and performance enhancement

Muscle glycogen is an important fuel during periods of prolonged exercise and a relationship between increasing exercise intensity and a reliance on muscle glycogen is evident. Fatigue has been directly related to depleting glycogen stores (Bergström &

Hultman, 1966, 1967; Bergström *et al.*, 1967). Hence, the post-exercise glycogen synthesis rate is essential in determining the time required for recovery. It has been shown that carbohydrate and wheat protein hydrolysate combined with an amino acid mixture (0.8 g/kg/hr and 0.4 g/kg/hr, respectively), administered to cyclists for a five hour period post-exercise, increases glycogen synthesis rates compared to administration of carbohydrate alone (van Loon *et al.*, 2000). Similarly, peak creatine kinase (CK) levels, as a result of initial muscle injury, were suppressed by wheat gluten hydrolysate in a dose-dependent manner *in vivo* (Koikawa *et al.*, 2009). Soy protein isolate is beneficial in muscle protein synthesis (MPS) following exercise. Consumption of the soy protein isolate was more effective than casein isolate but less effective than whey hydrolysate at increasing MPS both at rest and following resistance exercise (Tang *et al.*, 2009b). Calbet and MacLean (2002) suggested that carbohydrate and protein hydrolysates stimulate a synergistic insulin response, regardless of protein source. It was also found that the glucagon response depends on the increase in plasma amino acid composition, following protein solution ingestions and that pea and whey protein hydrolysates increased insulin to a greater extent and increased plasma amino acids more rapidly than cow's milk solution. It has been suggested that hydrolysates, particularly containing di- and tri-peptides, are absorbed more rapidly than either intact proteins or free form amino acids (Di Pasquale, 1997) which would support the use of protein hydrolysates for post-exercise recovery drinks as this would result in a greater increase in plasma amino acid concentration compared with the intact protein, over a two hour period (van Loon *et al.*, 2000b). The concentration of amino acids present in the blood regulates protein synthesis (Bohè *et al.*, 2003).

1.3.4.5. Other clinical applications

Protein hydrolysates represent an alternative to intact proteins and elemental (amino-acid based) formulas for the treatment of patients with various conditions. Phenylketonuria is a

disorder of amino acid metabolism, specifically, absence or deficiency of phenylalanine hydroxylase for the conversion of phenylalanine to tyrosine. The lack of this enzyme results in phenylpyruvic acid accumulation in the blood which has intellectual and neurological implications if left untreated. Protein hydrolysates free of phenylalanine have been used for the treatment of phenylketonuric infants, with positive results on physical growth and mental development (Acosta *et al.*, 1998; Berry *et al.*, 1976). Hydrolysates suitable for the treatment of phenylketonuria have been prepared from animal proteins including casein (Bickel *et al.*, 1954) and whey (Delvivo *et al.*, 2006). Plant protein hydrolysates low in phenylalanine have been studied to a lesser extent, however the potential of a low-phenylalanine soybean hydrolysate for dietetic purposes has been investigated (Yamashita *et al.*, 1976). In patients with chronic liver disease, complex alterations in the metabolism of proteins occurs and nutritional support is essential in the pathogenesis and treatment of this disease (Clemente, 2000). Patients with chronic liver failure have high plasma levels of aromatic amino acids (AAA) and methionine and low levels of branched-chain amino acids (BCAA) (Schenker & Beer, 1989; Morgan *et al.*, 1982). While casein hydrolysates are commonly used for nutritional applications in patients with chronic liver disease, a protein source with a higher level of BCAA is more desirable. Sunflower globulins have been suggested as excellent protein sources for the development of protein hydrolysates with high levels of BCAA (Bautista *et al.*, 1996). Sunflower protein hydrolysate is recommended for enteral, parenteral and oral nutrition of liver disease patients, being hypoallergenic, having low bitterness and providing a high Fischer ratio (BCAA:AAA) of approximately 75 (Bautista *et al.*, 2000).

1.3.5. Techno-functional properties of protein hydrolysates

1.3.5.1. Solubility

Solubility is the most important and generally the first techno-functional property examined during the development of new protein ingredients (Zayas, 1997) due to its considerable effect on other techno-functional properties (Vojdani, 1996; Kinsella & Melachouris, 1976). It has been proposed that reduction of the secondary structure of a protein and the enzymatic release of smaller polypeptide units are responsible for the increased solubility of hydrolysates compared to the original intact protein (Chobert *et al.*, 1988; Adler-Nissen, 1986). The solubility of a number of protein hydrolysates from agricultural crops has been studied. Barley protein hydrolysates showed highest solubility at strongly basic (pH 10 - pH 12) conditions (Yalcin & Celik, 2007). Similarly, Claver *et al.* (2005) found that the solubility of wheat protein hydrolysates was strongly influenced by pH, with lowest and highest solubility at pH 4 and 6, respectively. The use of rapeseed and other oilseed protein isolates is restricted due to their low solubility, which is a result of protein denaturation during industrial oil extraction (Vioque *et al.*, 2000). To improve solubility and functionality, protein isolates from oilseeds can be hydrolysed. It has been found that rapeseed hydrolysates exhibit > 90 % solubility at pH 5 - 9 (Yoshie-Stark *et al.*, 2008). Soy protein hydrolysates were found to be almost completely soluble (> 99 %) in the range of pH 2 – 9, whereas the intact protein had highest solubility at pH 9 (Chiang *et al.*, 1999). Tsumara *et al.* (2005) also demonstrated that the solubility of soy protein hydrolysates was pH-dependent. The production of hydrolysates that are soluble at acidic pH is essential for the supplementation of fruit juices and acidic drinks (Clemente, 2000; Mahmoud, 1994). In contrast to soy protein hydrolysates, okara protein isolates showed highest solubility at pH 12, with acid modified isolates enhancing solubility, thus increasing potential applications of okara protein as a food ingredient (Chan & Ma, 1999). Chan and Ma (1999) suggested that the low solubility of okara (a by-product of soymilk

manufacture) hydrolysates compared to soy hydrolysates is due to protein denaturation caused by severe heat treatments during soymilk manufacture.

1.3.5.2. Emulsifying properties

The ability of proteins to interact with lipids and form stable emulsions is essential to yield a stable food product. Rapeseed protein hydrolysates have higher emulsifying activity (at least 20 % greater) and stability than rapeseed protein isolates (Vioque *et al.*, 2000). Enzymatic hydrolysates of soy protein resulted in an increased emulsification activity (Barca *et al.*, 2000). Studies examining protein hydrolysates from different crop sources suggest that the emulsifying capacity of the hydrolysates is related to the degree of hydrolysis, with a low degree of hydrolysis (3 - 5%) increasing and a high degree of hydrolysis (~ 8 %) decreasing emulsifying capacity (Kong *et al.*, 2007; Vioque *et al.*, 2000; Achouri *et al.*, 1999). Ultrafiltered rapeseed protein hydrolysate was demonstrated to have greater emulsification stability compared to that of whole egg (Yoshie-Stark *et al.*, 2008) and wheat germ protein hydrolysates had higher emulsification capacity, activity and stability than bovine casein (Claver & Zhou, 2005). It is generally accepted that limited hydrolysis improves the emulsification properties of proteins by exposing hydrophobic amino acid residues (which may interact with oil), while the hydrophilic residues interact with water (Vioque *et al.*, 2000). Similarly, an increase in hydrophilicity as a result of acid modification has been shown to increase the emulsification activity index (EAI) of okara protein isolates (Chan & Ma, 1999).

1.3.5.3. Foaming

Foaming is of special interest in the food industry as it provides a desirable and unique texture to a range of aerated foods and beverages including ice-cream, bread, cakes, meringues, champagne and beer. It is essential that food foams are stable for consumer

acceptability, since consumer perception of quality is influenced by appearance. Wheat germ has been shown to have poor foaming properties (German *et al.*, 1985). However enzymatic treatment of wheat germ increases foam volume/height but decreases foam stability. The trend of increased foam volume being coupled with decreased foam stability has been reported in previous studies on rice bran protein hydrolysate and acid modification of okara protein hydrolysate (Zhang *et al.*, 2012; Chan & Ma, 1999). The absence of large protein components, which function to stabilise the foam, may contribute to the observed lack of foam stability (Claver & Zhou, 2005). In a similar study of wheat germ hydrolysates it was found that foaming capacity was increased at a degree of hydrolysis (DH) of 5 %, resulting in a 74 % increase in foam volume compared to the control. The foam was also most stable at DH of 5 %, with 40 % of foam volume sustained after 60 min. There was an inverse relationship between DH and foam stability, with stability in the order of DH 5 % > DH 10 % > DH 15 %. Similar to suggestions by Claver & Zhou (2005), the stability of the foams was attributed to the presence of larger component proteins and a partial hydrolysis, whereas a higher DH increases the number of polypeptide chains which do not have the ability to stabilise foams (Kong *et al.*, 2007). The study of both soy (Tsumara *et al.*, 2005) and rapeseed (Vioque *et al.*, 2000) protein hydrolysates produced comparable results. Regarding the effect of pH on foaming, barley hydrolysates had greater foam stability at basic pH values and very low stability at acidic pH (Yalcin *et al.*, 2008).

1.3.5.4. Gelation

The ability to manipulate the gel formation properties of a substance is important since gelation is desirable for the bakery and meat industries, but not for foods such as beverages and frozen deserts (Harper *et al.*, 1992). As a food ingredient, gelation is one of the most important techno-functional properties of soy protein, however soy protein hydrolysates

possess poor gel-forming ability (Babiker, 2000). Transglutaminase (TGase) is a polymeriser shown to be effective in improving gelling of proteins (Sakamoto *et al.*, 2006), which improved the gelling ability of soy protein hydrolysates (Fan *et al.*, 2005; Babiker, 2000) but the gels formed were inferior to the starting soy protein/isolate in terms of gel strength. It has been suggested that the reduced ability to form gels may be due to lower surface hydrophobicity and short peptide chain length of the hydrolysates (Fan *et al.*, 2005). Research has been carried out with a focus on manipulating conditions to give desired gelation properties. For example, it has been shown that sodium chloride (NaCl) concentrations greater than 0.2 M can accelerate gelation by sunflower protein hydrolysates but result in a gel of lower strength (Sanchez & Burgos, 1997). Addition of a polysaccharide such as guar gum can enhance the gel strength of canola protein isolates (Lèger & Arntfield, 1993) and protein concentration and pH have been identified as important factors influencing gel formation by canola protein isolate coupled with guar gum (Uruakpa & Arntfield, 2005). Hence, while the gel forming ability of protein hydrolysates from agricultural crops is not as strong as that from proteins, processes are available to improve gelation where necessary.

1.3.6. Safety of protein hydrolysates

The use of dietary proteins and protein hydrolysates in food products is generally allowed in European countries and has the status of 'generally regarded as safe' (GRAS) in the United States of America (US Food and Drug Administration, online article). In Europe, novel foods are defined as foods and food ingredients that have not been used for human consumption to a significant degree within the European Community before 15 May 1997 (Commission of the European Communities, online article). Safety evaluation by external independent experts and approval by competent authorities is necessary before a novel product is allowed on the market. Schaafsma (2009) proposed a decision tree that should be

used for determining the proposed safety assessment of protein hydrolysates and fractions thereof. Factors for consideration in deciding if a protein hydrolysate should follow procedure for novel foods include documented history of safe use, acceptable food grade hydrolysis process and the effect of intake on amino acid levels (Schaafsma, 2009). Hydrolysed proteins have a long history of safe use, but it is important to note that the majority of studies in this area look at animal-derived protein hydrolysates in infant feeding practices (Szajewska *et al.*, 2001; Decsi *et al.*, 1996; Halcken *et al.*, 1993). Some evidence has been produced regarding the sub-chronic toxicity of plant protein hydrolysates. Consumption of potato protein isolates is “well tolerated and without adverse effect” in Wistar rats, with parameters including body weight, body weight gain, mortality and organ weight remaining unchanged (Lynch *et al.*, 2012). Protein isolates from canola have also been reported to be safe, following a 13 week consumption trial in rats, being practically devoid of natural toxicants and environmental contaminants. The canola protein isolates had no effect on body weight, food consumption, clinical observations, motor activity or ophthalmic examinations (Mejia *et al.*, 2009). One particular consideration for the safety of protein hydrolysates and bioactive peptides is allergenicity. Most allergenic substances are protein-based compounds (Sarmadi & Ismail, 2010), and while hydrolysis breaks down proteins into low molecular weight peptides thus lessening the allergenic properties (Moure *et al.*, 2005), some hydrolysates may retain their parent protein allergenic effects (Hartmann *et al.*, 2007). Therefore, there is a need to further investigate the safety of plant-derived protein hydrolysates and bioactive peptides.

1.3.7. Conclusion

Protein hydrolysates derived from agricultural crops have exhibited antioxidant and ACE inhibitory potential *in vitro*, with low molecular weight fractions demonstrating greatest effects. There is substantial evidence which supports the ability of soy protein hydrolysates

to reduce CVD risk however hydrolysates obtained from other plant sources require further investigation. Protein hydrolysates show greater potential than intact protein to increase muscle glycogen and muscle protein synthesis and have also demonstrated potential in the treatment of clinical conditions, particularly sunflower hydrolysates which may be used in cases of chronic liver disease. In terms of techno-functional properties, hydrolysates with a low DH have desirable effects on emulsification, foaming and solubility properties. To conclude, protein hydrolysates from agricultural crops have demonstrated favourable bioactive and techno-functional properties that could be exploited for the development of functional foods. For nutraceutical development, clinical trials are necessary to confirm biological activity and safety, in addition to addressing issues such as stability during food processing, organoleptic issues and identifying the fate of plant-derived hydrolysates during passage through the gastrointestinal tract. Furthermore, protein hydrolysates from co-products of the plant processing industry should be investigated for their potential bioactive and techno-functional properties.

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1.4. Bioaccessibility of Functional Ingredients

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1.4.1. Abstract

Bioaccessibility is defined as the amount of a food constituent transferred to the micelle fraction after digestion in the gut, when compared with the original amount of the constituent present in the food. Bioaccessible constituents may be able to pass through the intestinal barrier and hence become bioavailable within the body. Bioaccessibility is commonly determined by *in vitro* methods simulating the human digestion and is assumed to be a good starting point for estimating potential bioavailability of a food constituent. The study of bioaccessibility of functional ingredients is necessary in addition to studies on their potential beneficial nutritional effects. This review discusses the benefits and limitations of methods used to assess bioaccessibility, in addition to factors affecting bioaccessibility, both dietary and physiological. Evidence regarding the bioaccessibility of specific functional ingredients, including carotenoids and flavonoids is especially highlighted. Potential approaches to enhance bioaccessibility of functional ingredients are also discussed.

1.4.2. Introduction

The amount of a food constituent that is available for absorption, following its release from the food matrix into the gastrointestinal (GI) tract, is termed bioaccessibility. Therefore, only food constituents that are released from the food matrix by digestive enzymes or bacterial microflora are bioaccessible. Bioavailability is linked to bioaccessibility, but refers to the fraction of a food constituent that is absorbed and available in the bloodstream for utilisation in the body.

Functional ingredients are becoming increasingly important in the food industry, in response to consumer demand for foods with added benefits to improve health, wellness and quality of life (Bleil, 2010). Increased life expectancy, high healthcare costs, advances in food science and technology and heightened awareness of health and nutrition among the general public are among the reasons why consumers are looking to the food industry for healthier versions of their favourite foods. Functional foods, nutraceuticals, designer foods and pharma-foods are all synonyms for foods that provide additional physiological benefits beyond their basic nutrition (Day *et al.*, 2009). The global nutraceutical market is rapidly expanding and set to be worth €23.8 billion in 2015, according to market analyst Freedonia (online article).

Plant extracts represent a unique source of bioactive ingredients, with the ability to promote human health benefits, however these effects are dependent on the efficient digestion and absorption of functional ingredients. The design of foods to encapsulate, protect and release bioactive components at specific locations is an area of increasing interest, with accurate methodology to assess bioaccessibility playing a crucial role in the development of such foods (Hur *et al.*, 2011)

Simulation of human digestion *in vitro* is commonly used as a tool to measure bioaccessibility, in fact *in vitro* digestion systems model the human stomach and small intestine, accounting for parameters such as pH, churning and enzymatic conditions. To allow for the complexity of the physiological process in the upper digestive tract more advanced models have been developed, that can account for other events such as gastric emptying and continuous changes in pH and secretion flow rates (Guerra *et al.*, 2012).

Assessment of the bioaccessibility of functional ingredients is critical in determining potential bioavailability and is essential in the design of foods claiming a health benefit due to the presence of bioactive ingredients. In studies where the bioaccessibility of functional ingredients is not measured, the significance of the results

for health is limited. This review aims to evaluate the methods used to assess bioaccessibility and factors that can affect bioaccessibility of functional food ingredients. A particular emphasis will be placed on specific functional ingredients such as carotenoids, flavonoids and hydroxycinnamic acids. Furthermore, possible means by which to enhance bioaccessibility will be detailed.

1.4.3. Bioaccessibility screening methods

The human digestive system is complex and comprises many processes occurring at different stages throughout the gastrointestinal tract, as shown in Figure 1. Briefly, mastication and enzymatic degradation (with salivary enzymes amylase, lingual lipase) occur in the mouth, producing a food bolus which is transported to the stomach by peristalsis (Guerra *et al.*, 2012). In the stomach, the food bolus is exposed to gastric juice comprising pepsin, gastric lipase and hydrochloric acid (HCl) and mechanically degraded by grinding and mixing. Chyme (small particles) is emptied into the duodenum section of the small intestine where it is neutralised with sodium bicarbonate (NaHCO₃). Enzymatic (bile and pancreatic enzymes (proteases, lipases, amylases)) and mechanical digestion (mixing) act to breakdown food constituents. Peristaltic waves then drive the movement of the digesta through the small intestine (Trendelenburg, 2006). Non-absorbed material reaches the large intestine, where water and electrolyte absorption occurs, bile salts are reabsorbed, faeces are formed, stored and eliminated and polysaccharides and proteins are fermented by colonic microbiota (Guerra *et al.*, 2012).

1.4.3.1 In vivo digestion methods

Measurements *in vivo* actually determine the amount of a food/nutrient that is absorbed, i.e. bioavailability. *In vivo* approaches are considered the “gold standard” of bioavailability assessment, but disadvantages of such approaches include the expense and

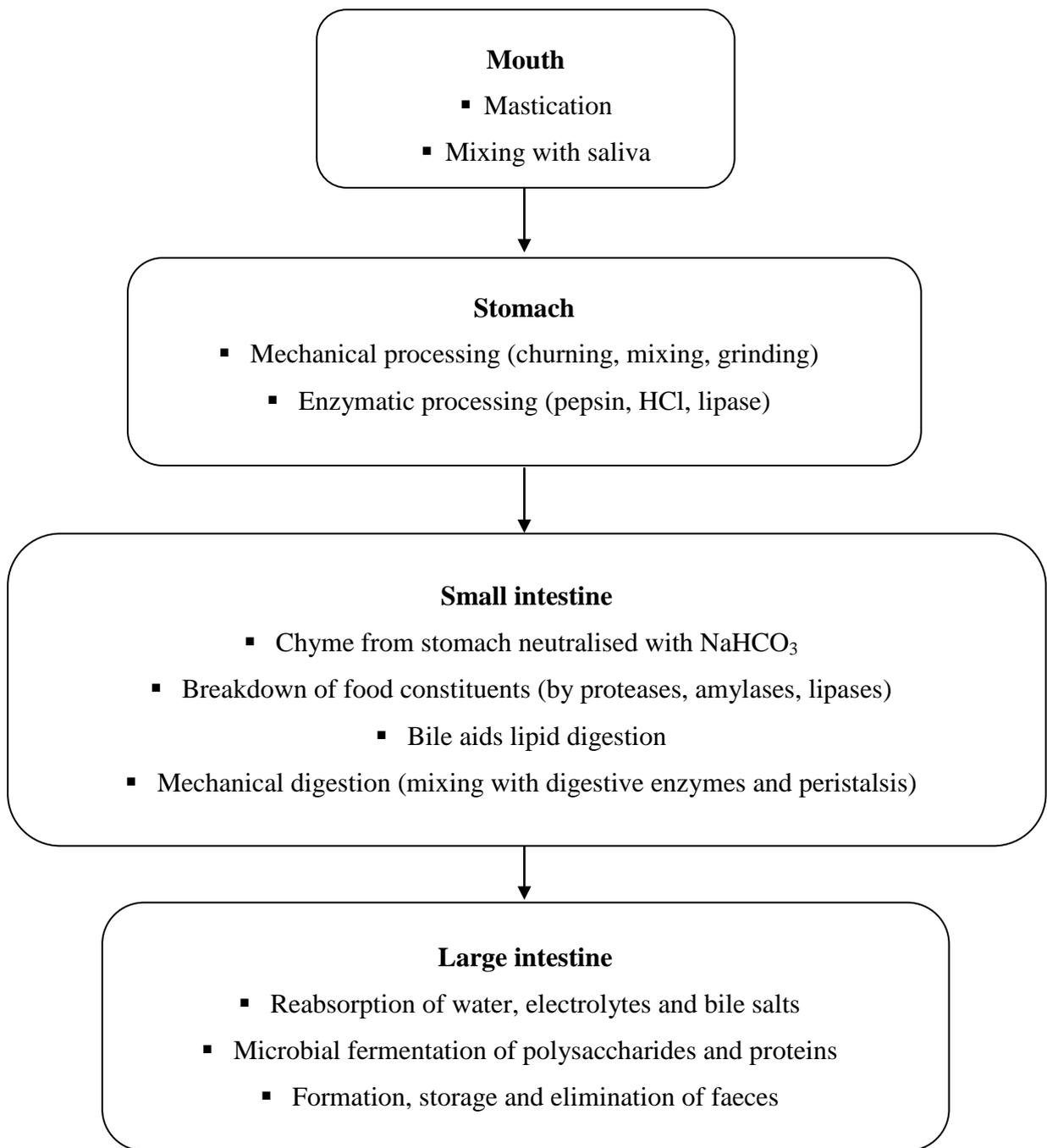


Figure 1: The main digestive processes in the human gastrointestinal tract.

limited throughput (Table 1).

Digestion *in vivo* has been carried out for a number of compounds that have potential applications as functional food ingredients, including carotenoids and tocopherols (Granado *et al.*, 2006), lutein (Granado-Lorencio *et al.*, 2010), naringenin and chlorogenic acid (Bugianesi *et al.*, 2004).

Common *in vivo* methods to determine the amount of nutrients and bioactive compounds absorbed include balance studies and tissue concentration measurements. Balance studies measure the absorbed amount of a nutrient or bioactive compound by measuring the difference between the consumed and excreted amounts (Fernandez-Garcia *et al.*, 2009). Tissue concentration measurements are more frequently used; this method involves measuring the change in plasma/serum concentration of the nutrient or bioactive compound (Fernandez-Garcia *et al.*, 2009). *In vivo* bioavailability of a number of bioactive ingredients including carotenoids and tocopherols (Granado *et al.*, 2006), lutein (Granado-Lorencio *et al.*, 2010) and grape seed procyanidin extract (Serra *et al.*, 2010) has been determined by tissue concentration measurements.

1.4.3.2. *In vitro* gastrointestinal digestion methods

Various *in vitro* digestion models have been developed, as a faster, lower-cost alternative to *in vivo* methods (Table 1). While measuring bioaccessibility by *in vitro* methods has a number of advantages, there are also limitations to this approach, which are mainly related to the inability to exactly mimic the *in vivo* digestion process, as highlighted in Table 1. *In vitro* digestion models can be either static or dynamic. Static models do not account for the physical processes that occur *in vivo*, such as mixing, peristalsis and changes in conditions over time (Fernandez-Garcia *et al.*, 2009). Contrastingly, dynamic models mimic these physical processes and allow for factors including gastric emptying, continuous changes in pH and secretion flow rates (Guerra *et al.*, 2012). Static *in vitro*

Table 1: Benefits and limitations of *in vivo* and *in vitro* methods for bioavailability and bioaccessibility measurement.

	<i>In vivo</i>	<i>In vitro</i>
Benefits	<ul style="list-style-type: none">▪ “Gold standard” of assessment (Failla <i>et al.</i>, 2008)▪ Allows selection of subjects from target population of intended use (Fernandez-Garcia <i>et al.</i>, 2009)▪ Provide direct data on bioavailability (Guerra <i>et al.</i>, 2012)	<ul style="list-style-type: none">▪ High-throughput (Fernandez-Garcia <i>et al.</i>, 2009)▪ Provides information about each digestion step (Fernandez-Garcia <i>et al.</i>, 2009)▪ Lower cost (Fernandez-Garcia <i>et al.</i>, 2009)▪ Standard method technically simple (Failla <i>et al.</i>, 2008)
Limitations	<ul style="list-style-type: none">▪ Lower throughput (Fernandez-Garcia <i>et al.</i>, 2009)▪ Ethical considerations (Fernandez-Garcia <i>et al.</i>, 2009)▪ Expensive (Fernandez-Garcia <i>et al.</i>, 2009)	<ul style="list-style-type: none">▪ Complex physiology of intestine not fully reproduced (Fernandez-Garcia <i>et al.</i>, 2009)▪ Do not account for feedback mechanisms, resident microbiota, immune system, hormonal controls (Guerra <i>et al.</i>, 2012)▪ Large intestinal phase generally not included (Failla <i>et al.</i>, 2008)

digestion models (also known as biochemical models) are the most common and usually mimic both gastric and intestinal digestion through the use of pepsin and pancreatin and bile salts, respectively. Temperature (37 °C), shaking and the chemical and enzymatic composition of saliva, gastric juice and bile juice are the key features of the gastrointestinal (GI) *in vitro* digestion model. Investigators may choose to use a two (gastric and duodenal) or three (oral, gastric, duodenal) phase digestion system, depending on the purpose of the study. For solid foods, oral processing is difficult to simulate and methods used include a “chew and spit” process where possible or homogenisation (Wickham *et al.*, 2009). For liquid foods, this phase is not necessary, but salivary amylase may be added (Wickham *et al.*, 2009). Digestion in the stomach and small intestine is far from complete, particularly the digestion of plant foods because plant cell walls are not degraded in the upper GI tract. Within static models there can be considerable variations in experimental conditions; differences in pH used for gastric and duodenal phases, addition of specific enzymes including α -amylase and lipase to digest foods with a high starch or fat content, respectively, and addition of antioxidants to limit oxidation (van Buggenhout *et al.*, 2010). At present, no standardised *in vitro* digestion model exists and variations in approaches to *in vitro* models have been detailed elsewhere (Hur *et al.*, 2011). Since the luminal (external) environment influences the rate, extent and site where a nutrient becomes available for digestion, dynamic models incorporating physical conditions and changes in luminal conditions over time have been developed. A dynamic GI model was developed by TNO Nutrition and Food Research (The Netherlands), which closely mimics the complex *in vivo* digestion process. On the basis of studies on human volunteers, parameters such as pH, temperature, peristaltic mixing and transit, biliary and pancreatic secretions are replicated. The TNO system can also account for different life stages, different food intakes and physiological and pathological conditions (Blanquet *et al.*, 2004), however the high cost associated with this system limits its use. A number of

studies have employed this dynamic GI model to determine the bioaccessibility of various compounds including xanthophylls and hydroxycinnamic acids (Blanquet-Diot *et al.*, 2009; Hemery *et al.*, 2010). A study on determining xanthophyll and carotenoid bioaccessibility highlighted that the TNO system allowed the identification of parameters influencing bioavailability including food matrix, nature, isomeric conformation and the digestive compartment (Blanquet-Diot *et al.*, 2009). Hemery *et al.* (2010) investigated the bioaccessibility of hydroxycinnamic acids in breads produced with bran and found that the dynamic TNO model provided results comparable with a previous *in vivo* study (Hemery *et al.*, 2010; Kern *et al.*, 2003). Contrastingly, bioaccessibility results obtained from studies using static and TNO models have been found to differ. For example, β -carotene and lycopene from tomato were found to be more bioaccessible using the TNO model compared to results of a study using static conditions, suggested to be attributable to the degradation of carotenoids in the gastric phase of the static digestion model (Blanquet-Diot *et al.*, 2009; Granado-Lorencio *et al.*, 2007). Similarly, discrepancies in bioaccessibility of food contaminants, including lead and mercury, measured by static and dynamic digestion models have been reported (Torres-Escribano *et al.*, 2011).

The coupling of *in vitro* digestion models with experiments in intestinal caco-2 cells in culture allows host responses to be accurately replicated. Caco-2 cells are a human intestinal cell line, which mimic intestinal absorptive epithelium when grown in a monolayer and represent the best available cell culture model of absorptive small intestinal enterocytes (Feruzza *et al.*, 2012). Combining the TNO dynamic *in vitro* digestion model with a caco-2 cell model has been shown to produce results consistent with *in vivo* findings on the bioavailability of lycopene and α -tocopherol (Deat *et al.*, 2009).

1.4.3.3. Comparison of *in vivo* and *in vitro* digestion methods

Comparing *in vivo* and *in vitro* approaches to measuring bioaccessibility has been studied, as detailed in Table 2 (Granado *et al.*, 2006; Granado-Lorencio *et al.*, 2010). A study of bioaccessibility of carotenoids and tocopherols from broccoli highlighted that *in vivo* and *in vitro* approaches can give conflicting results (Granado *et al.*, 2006). High performance liquid chromatography (HPLC) of digestates formed by *in vitro* gastrointestinal digestion found that lutein and β -carotene have similar stability, whereas *in vivo* tissue concentration measurements showed that lutein levels significantly increased following digestion but β -carotene levels did not (Granado *et al.*, 2006). A similar study of lutein ester-fortified fermented milk yielded consistent results in both approaches and the importance of *in vitro* bioaccessibility results as predictors of *in vivo* bioavailability was noted (Granado-Lorencio *et al.*, 2010). It is essential that both approaches should be considered complementary, not inter-changeable, as *in vitro* methods do not account for host-related factors such as biological actions in the body (Granado *et al.*, 2006).

1.4.4. Factors affecting bioaccessibility of functional ingredients

There are a large number of factors than influence the bioaccessibility/bioavailability of functional ingredients, either directly or indirectly (by altering the content of the bioactive ingredient). These include dietary, physiological and processing factors, as detailed in Table 3.

1.4.4.1. Dietary factors

Disrupting the food matrix and releasing the constituents is the first step of absorption and the influence of food matrix on the bioaccessibility of food components has been well documented. It has been reported that β -carotene is less bioavailable from vegetables than from a simpler matrix (van het Hof *et al.*, 2000). In addition, lutein and β -carotene are less

Table 2: *In vivo* and *in vitro* studies measuring the bioaccessibility of potential functional ingredients.

Study Type	Ingredient	Aim	Study Design	Comments	Reference
<i>In vivo & in vitro</i>	Carotenoids and tocopherols from broccoli	To determine bioavailability of carotenoids and tocopherols from broccoli and compare <i>in vivo</i> and <i>in vitro</i> approaches	<ul style="list-style-type: none">• <i>In vivo</i>: 7-day intervention trial in healthy volunteers. 200 g/day broccoli consumed, blood samples taken• <i>In vitro</i> : Static gastrointestinal digestion of broccoli• Followed by HPLC analysis of either blood samples or digestates	• Results showed that <i>in vivo</i> and <i>in vitro</i> approaches are complementary, not interchangeable	Granado <i>et al.</i> , 2006
<i>In vivo & in vitro</i>	Lutein	To assess bioavailability of lutein from lutein ester-fortified fermented milk	<ul style="list-style-type: none">• <i>In vivo</i>: Healthy volunteers consumed lutein ester-fortified fermented milk, total dose equalling 8 or 16 mg lutein (single-dose study) or 60 and 120 mg lutein (multiple-dose study). Fasting blood samples collected• <i>In vitro</i> : Static gastrointestinal digestion of lutein ester-fortified fermented milk• Followed by HPLC analysis of either blood samples or digestates	• <i>In vitro</i> models useful to predict <i>in vivo</i> responses	Granado-Lorencio <i>et al.</i> , 2010
<i>In vitro</i>	Carotenes and xanthophylls	To determine the digestive stability of carotenes and xanthophylls found in tomatoes	<ul style="list-style-type: none">• <i>In vitro</i>: dynamic TNO gastrointestinal tract model (TIM) used to digest test meals• HPLC analysis of digestates from each digestive compartment	• Use of a dynamic <i>in vitro</i> model allowed identification of parameters influencing bioavailability of carotenoids	Blanquet-Diot <i>et al.</i> , 2009
<i>In vitro</i>	Ferulic acid, sinapic acid, <i>p</i> -coumaric acid	Measurement of bioaccessibility of phenolic acids in breads made from processed bran	<ul style="list-style-type: none">• <i>In vitro</i>: dynamic TNO gastrointestinal tract model (TIM) used to digest breads	• Results correlated well with a previous <i>in vivo</i> study	Hemery <i>et al.</i> , 2010

bioaccessible from green leafy vegetables than from other vegetables (van het Hot *et al.*, 1999). There is mixed evidence regarding the effect of different food matrices on *in vivo* bioavailability; genistein is more bioavailable from a liquid matrix, than a solid matrix whereas daidzein bioavailability is not influenced by food matrices including cookies, juice and chocolate bars (de Pascual-Teresa *et al.*, 2006).

In addition to food matrix, dietary components consumed with phenolic compounds can greatly affect their bioaccessibility. It is consistently reported that the presence of lipid increases the bioaccessibility of potential functional food ingredients, including carotenoids (Hornero-Mendez *et al.*, 2007) and isoflavones (Walsh *et al.*, 2003). This is possibly attributable to the ability of foods containing lipids and proteins to increase plasma cholecystokinin (CCK) and induce the release of bile into the duodenum (Otsuki, 2002), thus increasing bioavailability. It has also been suggested that non-competitive interactions, where soluble and insoluble complexes are formed, may account for this observed effect, with products of fat digestion solubilising fat soluble vitamins and carotenoids, thus enhancing absorption (Gibson, 2007). It is important to note that the presence of non-absorbable fat soluble compounds such as sucrose polyester (a fat replacer) can decrease plasma levels of bioactive compounds like carotenoids (Westrate & van het Hof, 1995; Schlagheck *et al.*, 1997). The incorporation of carotenoids released from the food matrix into the sucrose polyester rather than the micelles from dietary fat is a possible explanation for this. In contrast to the presence of fat and protein, dietary fiber can reduce bioavailability of genistein (Tew *et al.*, 1996). There is conflicting evidence regarding the relationship between fiber and daidzein bioavailability. Dietary fiber consumption has been shown to stimulate equol (daidzein metabolite) production (Lampe *et al.*, 1998; Rowland *et al.*, 1999). However, it has also been reported that equol producers have lower dietary fiber intake (Gardana *et al.*, 2009). Binding of bile acids by lignin and pectin and the retention of water and formation of viscous solutions in the gut

by pectins, gums and psyllium both contribute to the reduced absorption of fat-soluble vitamins, carotenoids and nutrients (Gibson, 2007).

1.4.4.2. Physiological factors

Host-related factors that can influence the bioaccessibility of dietary components include intestinal (enzyme activity, colonic microflora) and systemic (gender, age, pathologies and disorders, genetics) factors (D'Archivio *et al.*, 2010). Intestinal digestion and absorption of dietary components is largely influenced by atrophic gastritis and hypochlorhydria (decreased secretion of hydrochloric (HCl) acid). The resultant alteration in pH levels can impair absorption of vitamins and minerals, including iron, calcium, magnesium and vitamin C (Nolan *et al.*, 2012). Bacterial overgrowth is also associated with atrophic gastritis and can alter the intestinal mucosal integrity, increasing intestinal permeability and reducing nutrient absorption (Gibson, 2007). Infection with salmonella, rotavirus, malaria and *Giardia lamblia* can reduce transit time, decreasing the time for extensive solubilisation in the gastrointestinal tract and compromising absorption (Gibson, 2007). This phenomenon has been demonstrated in women; following consumption of 1.2 mg total isoflavone/kg, a rapid gut transit time coupled with low fecal genistein disappearance was associated with greater genistein bioavailability, by urinary genistein excretion (Zheng *et al.*, 2003). Furthermore, there was a genetic influence of this effect, which was observed in Asian but not Caucasian subjects and daidzein disappearance significantly differed between the two ethnic groups. The authors suggested this ethnic effect may be due to variation in gut microflora as a result of ethnic differences in diet e.g. red meat and soy food (Zheng *et al.*, 2003).

Potential functional ingredients such as isoflavones and γ -tocopherol are more bioavailable in women (Nielsen & Williamson, 2007; Granado *et al.*, 2006). Higher urinary excretion of isoflavone conjugates in women was recorded in a long-term feeding

study, followed by an increase in urinary excretion of equol, which remained unchanged in men (Lu & Anderson, 1998). While serum lutein levels were increased in both male and female subjects following broccoli consumption (200 g) for 7 days, serum γ -tocopherol levels were increased (by ~ 23 %) in women only (Granado *et al.*, 2006). The response to xenobiotics, both dietary phytochemicals and drugs, can be influenced by genetic variations arising from polymorphisms of genes coding for phase 1 and phase 2 metabolising enzymes (Holst & Williamson, 2004). Resulting increase or decrease in enzyme activity, or the absence of the enzyme, can lead to altered bioavailability (Holst & Williamson, 2004). Disease states can also influence bioavailability, particularly of minerals, either directly, by increasing or decreasing physiological requirements or indirectly, by altering intestinal function and metabolic pathways. Such diseases include chronic renal failure, diarrhoea and vomiting, Crohn's disease, Wilson's disease (Cu) and hyper- and hypo-parathyroidism (Ca) (Fairweather-Tait, 1996).

1.4.4.3. Processing factors

The most extensively studied processing factors influencing bioaccessibility and bioavailability are thermal processing, homogenisation, storage and freezing. These factors influence bioaccessibility indirectly, by altering the content of the dietary component.

The effect of domestic cooking on *in vivo* bioavailability of tomato polyphenols was determined by Bugianesi *et al.* (2004). Plasma naringenin and chlorogenic acid levels were increased following consumption of cooked tomatoes. It was suggested that the increase in naringenin bioavailability was due to the ability of heat treatments to break down interactions between naringenin and insoluble polyesters (constituents of tomato fibre) in the ripe tomato fruit (Bugianesi *et al.*, 2004). A more recent study by Mazzeo *et al.* (2011) showed that steaming (for minimum time to reach tenderness for adequate

palatability) of frozen vegetables increased bioaccessibility and limited carotenoid depletion, whereas boiling of frozen vegetables resulted in a general loss of carotenoids, phenolic compounds and total antioxidant capacity (Mazzeo *et al.*, 2011). Severe boiling of tomatoes resulted in increased lycopene bioaccessibility, although this increase was non-significant (van het Hof *et al.*, 2000b). In the same study, consumption of mildly and severely homogenised tomatoes significantly increased plasma lycopene levels by 0.03 and 0.04 $\mu\text{mol/L}$, $P < 0.05$, compared to non-homogenised tomato samples (van het Hof *et al.*, 2000b).

Just as the duration and method of cooking influenced bioaccessibility outcomes, the effect of storage on the phenolic content or bioaccessibility of different foods depends on the storage conditions, as summarised in Table 3. Storage conditions of red raspberries simulating those found in the supermarket (4 °C, 3 days) and in the home (18 °C, 1 day) did not effect the anthocyanin content (Mullen *et al.*, 2002). In the same study, it was found that freezing red raspberries increased the coumaric acid concentration. Similarly, storage of lutein fortified frankfurters for 22 days at 4 °C did not affect lutein content (Granado-Lorencio *et al.*, 2010b). However, cold storage of apples increased catechin levels (Napolitano *et al.*, 2004). Storage of sweet potato roots in a pit at temperature 17 – 21 °C and relative humidity (RH) 90 – 100 % had higher β -carotene contents than roots stored at ambient conditions (24 - 27 °C, RH 68 – 100 %), with the study providing evidence that roots stored in lower temperatures retain β -carotene levels to a greater extent and maintains *in vitro* bioaccessibility (Tumuhimbise *et al.*, 2010).

In light of this evidence, it is clear that the influence of processing on bioaccessibility is complex, with varying results for different functional ingredients. Domestic cooking, and steaming rather than boiling to avoid leaching, homogenisation and storage at lower temperatures can increase the content and bioaccessibility of certain phenolic dietary components.

Table 3: Factors influencing bioaccessibility of functional ingredients.

Factors	Functional ingredient	Influence	Reference
<i>Dietary</i>			
Food matrix	Carotenoids	Lower bioavailability of β -carotene from vegetables than from a simpler matrix (e.g. salad dressing)	van het Hof <i>et al.</i> , 2000
Protein and lipids	Soy isoflavones	Earlier peak in blood genistein concentrations from liquid matrix (versus solid matrix). Daidzein bioaccessibility unaffected by food matrix	de Pascual-Teresa <i>et al.</i> , 2000
	Isoflavones	Increased bioavailability from foods containing protein and lipids,	Walsh <i>et al.</i> , 2003
	Carotenoids	Unabsorbable fat-soluble compounds (e.g. sucrose polyester) reduced absorption	Westrate & van het Hof, 1995; Schlagheck <i>et al.</i> , 1997
Fibre	Carotenoids	Addition of cooking oil increased bioaccessibility	Hornero-Mendez <i>et al.</i> , 2007
	Soy isoflavones	Decreased bioavailability of daidzein by a fibre rich diet	Tumuhimbise <i>et al.</i> , 2010
<i>Physiological</i>			
Gut transit time	Isoflavones	Bioavailability increased by rapid gut transit time and low faecal digestion rates	Nielsen & Williamson, 2007
<i>Processing</i>			
Thermal Processing	Lycopene	Severe heat treatment (boiling for 1 hr @ 100 °C) of tomatoes increased bioavailability	van het Hof <i>et al.</i> , 2000b
	Naringenin & chlorogenic acid	Domestic cooking of tomatoes (15 min @ 100 °C) significantly increased naringenin and chlorogenic acid plasma levels	Bugianesi <i>et al.</i> , 2004
	Polyphenols & carotenoids	Steaming increased bioaccessibility of polyphenols & limited depletion of carotenoids. Boiling negatively influenced bioaccessibility of carotenoids and phenolic compounds	Mazzeo <i>et al.</i> , 2011
Homogenisation Storage	Lycopene	Increased bioavailability	van het Hof <i>et al.</i> , 2000b
	Lutein	Storage of lutein enriched frankfurters (22 days @ 4 °C) had no effect	Granado-Lorencio <i>et al.</i> , 2010
	β -carotene	Storage of sweet potato roots in dark, ambient (24-27 °C, RH 68-100 %) conditions had lowest bioaccessibility	Tumuhimbise <i>et al.</i> , 2010
	Anthocyanins Catechin	Storage of red raspberries (3 days @ 4 °C and 24 h @ 18 °C) had no effect Storage (4 months @ 2 °C) increased catechin conc. in apples	Mullen <i>et al.</i> , 2002 Napolitano <i>et al.</i> , 2004
Freezing	Coumaric acid	Present at higher conc. in frozen fruit	Mullen <i>et al.</i> , 2002

1.4.5. Bioaccessibility of functional food ingredients

1.4.5.1. Carotenoids

Carotenoids perform many functions and actions in all living organisms including animals and microorganisms, despite being conventionally considered as plant pigments responsible for the yellow, orange and red colouration of flowers and fruit. Carotenoids are crucial for photosynthesis, but have also been associated with the prevention of human diseases including cancer (Britton, 1995). Over 600 carotenoids have been identified, 40 of which are present in the human diet (Yonekura & Nagao, 2007). However, carotenoids cannot be produced by animals, therefore to play a role in human health, they must be consumed in the diet, and bioaccessibility is crucial. Only 14 carotenoids and some of their metabolites have been identified in blood and tissues, including carotenes (lycopene, β -carotene, α -carotene) and xanthophylls (lutein, β -cryptoxanthin, zeaxanthin) (Yonekura & Nagao, 2007). Sweet potatoes, tomatoes, bell peppers, carrots, spinach and kale are some of the commonly consumed sources of carotenoids. Absorption of carotenoids is initiated by disruption of the food matrix to release the carotenoids for incorporation into lipid droplets in the stomach. Bile salts, phospholipids, dietary lipids and their hydrolysis products produce mixed micelles where the carotenoids are transferred to, from the lipid droplets. This step is followed by absorption by the intestinal cells, packing into the chylomicrons and secretion to the lymphatic system (Yonekura & Nagao, 2007).

Much evidence has been carried out investigating the bioaccessibility of carotenoids, which has been extensively reviewed elsewhere (Rodriguez-Amaya, 2010), and it is generally accepted that *in vitro* simulated gastric and small intestinal digestion coupled with experiments in differentiated caco-2 cells, as previously discussed, represents a valid model for the initial screening of the bioaccessibility of carotenoids (Rodriguez-Amaya, 2010). This model has been used to measure the carotenoid bioaccessibility of bell and chilli peppers and raw and cooked carrots (O'Sullivan *et al.*,

2010; Aherne *et al.*, 2010). A study by Goni *et al.* (2006) details the bioaccessibility of carotenoids from fruits and vegetables, through the use of a method incorporating both *in vitro* enzymatic digestion (small intestine bioaccessibility) and colonic fermentation (large intestine bioaccessibility) and states the large intestine may be a significant site of carotenoid absorption in the gut (Goni *et al.*, 2006). Results found that 91 % of the lutein, lycopene and β -carotene found in fruits and vegetables are available in the gut following the entire digestion process (Aherne *et al.*, 2010b). Factors affecting the bioaccessibility of functional ingredients have been described in the previous section. Briefly, it has been shown that the location of carotenoids in the food matrix, carotenoid interactions and geographical location of production of vegetables play a role (Aherne *et al.*, 2010b; Aherne *et al.*, 2009). Further to this, the factors influencing bioavailability and bioconversion of carotenoids have been summarised by the mnemonic “SLAMENGHI”; **s**pecies of carotenoid, **m**olecular linkage, **a**mount of carotenoids consumed in a meal, **m**atrix of the food, **e**ffectors of absorption and bioconversion, **n**utrient status of the host, **g**enetic factors, **h**ost-related factors, **m**athematical interactions (West & Castenmiller, 1998).

It has been repeatedly reported that xanthophylls are more bioaccessible than β -carotene, assessed by both static *in vitro* digestion models and the dynamic TNO model (Granado-Lorencio *et al.*, 2010; Blanquet-Diot *et al.*, 2009; Kean *et al.*, 2008). Luminal stability of carotenoids may be affected by different levels of hydrophobicity influencing transfer from lipid droplets to the mixed micelles (Blanquet-Diot *et al.*, 2009). Digestive stability and subsequent bioavailability may be explained by differences in localisation in the lipid droplets (Borel *et al.*, 1996). It is important to note the methodology used in these studies do not incorporate *in vitro* colonic fermentation to assess large intestine bioaccessibility. This is crucial given the findings discussed previously where β -carotene was found to be released from the food matrix predominantly in the large intestine (Goni

et al., 2006). Thus, the choice of study design and methodology can have a major impact on the conclusions made regarding the bioaccessibility of carotenoids.

1.4.5.2. Flavonoids and other secondary plant metabolites

Secondary plant metabolites are produced from phenylalanine via the phenylpropanoid pathway, as shown in Figure 1, and comprise flavonoids, hydroxycinnamic acids, coumaroids and stilbenoids.

There are over 4,000 identified flavonoids of different classes; flavanols, flavones, flavanones, isoflavones, catechins and anthocyanins. Flavonoids can be found widely dispersed in plant foods, particularly berries, broccoli and kale, and other commonly consumed products such as chocolate and green tea. Flavonoids, except flavan-3-ols, in plants are bound to sugars, existing as glycosides (Del Rio *et al.*, 2010). The chemical structure of the sugar side chains to which the aglycones are bound is one of the most important factors affecting bioavailability (Koli *et al.*, 2010). During absorption, intestinal glycosidases rapidly hydrolyse flavonoid glycosides (Liu & Hu, 2002). This step is crucial in determining the intestinal fate of glycosides, as their uptake is limited by slow passive diffusion, poor uptake by the glucose transporter SGLT1 and the presence of an efflux carrier for glycosides (Liu & Hu, 2002). Contrastingly, flavonoid aglycones are rapidly absorbed (Liu & Hu, 2002) and conjugated by phase 2 enzymes, such as UDP-glucuronyltransferase in the intestine (Crespy *et al.*, 1999). This is followed by excretion in the urine or the bile. After biliary excretion, flavonoid conjugates are hydrolysed in the lower intestine by bacteria, resulting in flavonoid aglycones reabsorption and enterohepatic recirculation (Liu & Hu, 2002; Crespy *et al.*, 1999). Recently, Liang *et al.* (2012) reported only 5 % recovery of the anthocyanin glucosides cyanidin-3-rutinoside and cyanidin-3-glucoside following *in vitro* digestion (Liang *et al.*, 2012). This low bioaccessibility was postulated to be due to a few possible mechanisms – high instability

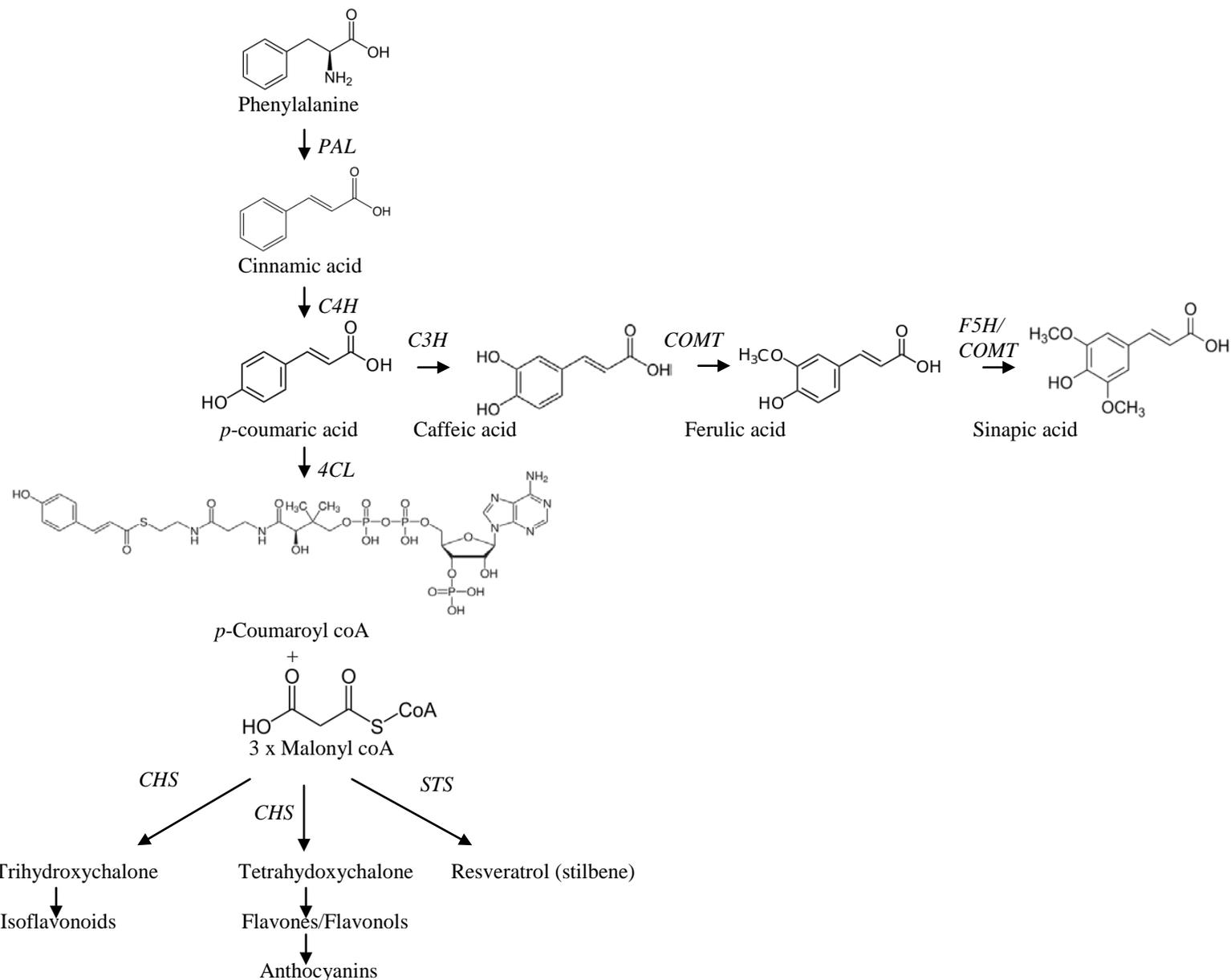


Figure 1: The phenylpropanoid pathway for the production of secondary plant metabolites including hydroxycinnamic acids and flavonoids. Enzymes are abbreviated as follows: PAL, phenylalanine lyase; C4H, cinnamate-4-hydroxylase; C3H, *p*-coumaroyl shikimate/quinate 3-hydroxylase; COMT, caffeic acid/5-hydroxyferulic acid O-methyltransferase; F5H, ferulate-5-hydroxylase; 4CL, 4-coumarate CoA ligase; CHS, chalcone synthase; STS, stilbene synthase.

of the compounds in the mildly alkaline conditions of the small intestine and degradation of anthocyanins to smaller phenolic compounds, possibly hydroxybenzoic acids (Liang *et al.*, 2012). The large change in pH from the acidic gastric environment to the mildly alkaline intestinal environment was also considered to be accountable for the 58.1 % loss in anthocyanins and overall 56 % loss of total polyphenols during the *in vitro* digestion of grapes (Tagliazucchi *et al.*, 2010). In contrast to anthocyanins, there is a general consensus that isoflavones are readily absorbed and reach peak concentrations within a few hours of ingestion. Rodriguez-Roque *et al.* (2013) reported 36 % bioaccessibility of total isoflavones from soymilk, with glycosides being more bioaccessible (27 – 42 %) than aglycones (15 – 17 %) (Rodriguez-Roque *et al.*, 2013). These values are relatively conservative, considering genistein bioaccessibility in the small intestine was found to range from 79.55 – 92.11 % depending on fat content in enriched custards (Sanz & Luyten, 2007); genistein bioaccessibility was only found to be 17 % by Rodriguez-Roque *et al.* (2013). The *in vitro* digestion methods used were very similar, however Sanz & Luyten (2007) used pH 2.5 and an incubation time of 60 min for gastric digestion, whereas Rodriguez-Roque *et al.* (2013) used a slightly lower pH of 2, with double the incubation period (120 min), which may have led to the discrepancies in findings between the studies. Further evidence for the high recovery and bioaccessibility of genistein is provided by Simmons *et al.* (2012). The authors formulated soy pretzels using raw soy ingredients that demonstrated 133.8 % recovery of genistein (Simmons *et al.*, 2012). There is much evidence available detailing the bioaccessibility of flavonoids, with isoflavones being the most bioavailable and anthocyanins and flavon-3-ols poorly absorbed (Corcoran *et al.*, 2012). Extensive biotransformation by methoxylation, glucuronidation and sulfation suggests that bioactivity of flavonoids may be due to these metabolites rather than the parent or free forms (Corcoran *et al.*, 2012). Proanthocyanidins and other flavonoid polymers must be broken down, primarily in the colon, to monomeric

and dimeric units for absorption. Therefore, it has been suggested that *in vitro* methods to assess flavonoid bioaccessibility give limited information, unless they include colonic fermentation processes, as in the TNO model (Etcheverry *et al.*, 2012). It has been reported that the fermentation of flavonoids from tea with human fecal bacteria results in the biotransformation of polyphenols and the production of metabolites that have significant bioactive effects (Lee *et al.*, 2006).

Hydroxycinnamic acids are hydroxy derivatives of cinnamic acid. The most common hydroxycinnamic acids are ferulic, coumaric and caffeic acids, present predominantly in cereal grains (ferulic, *p*-coumaric) and coffee (ferulic, caffeic), usually in the bound form. Cleavage, release and absorption of hydroxycinnamic acids may occur mainly in the small intestine (Kern *et al.*, 2003). Bacterial enzymes in the large intestine can further release hydroxycinnamic acids (Lee *et al.*, 2006), but the evidence regarding this step is inconclusive with some studies in rats (Adam *et al.*, 2002) and humans (Kern *et al.*, 2003) indicating little or no absorption in the large intestine, perhaps due to limited access of the digestive enzymes to the phenolic substrate (Adam *et al.*, 2002). Esterified hydroxycinnamic acids found in food are metabolised extensively following ingestion. Conjugated hydroxycinnamic acids, glucuronidated and predominantly sulphated forms, are primarily found in plasma and urine, while free acids are present at low levels (Wong *et al.*, 2010). Sulfotransferase 1A1 (SULT1A1) and UDP-glucuronosyltransferase 1A9 (UDP1A9) are the most active enzymes for hydroxycinnamic acid sulphation and glucuronidation, respectively (Wong *et al.*, 2010). Due to the polarity and water solubility of hydroxycinnamic acids, these phenolics are readily excreted in the urine and higher urinary excretion levels than plasma levels suggests these phenolics, and metabolites, do not accumulate in organs and tissues, and are rapidly filtered by the kidneys (Wang *et al.*, 2012).

Ferulic acid has shown low bioaccessibility (< 1 %) from wheat fractions (aleurone, bran, flour) and bread *in vitro*, which increased to ~ 60 % bioaccessibility following the addition of free ferulic acid to the flour (Mateo Anson *et al.*, 2009). Using the TNO intestinal model, bioaccessibility of ferulic, sinapic and *p*-coumaric acids was measured in white, brown and bran breads. Only the free and conjugated hydroxycinnamic acids were bioaccessible. The amount of bioaccessible hydroxycinnamic acids was higher in whole-grain and bran bread than in white bread and bran dry-fractionation increased the amounts of bioaccessible phenolic acids. Ultra-fine grinding of the bran decreased particle size of the cell-wall fragments, increased particle surface area and interactions between GI fluids and free and conjugated phenolic acids in the cell walls (Hemery *et al.*, 2010). It is generally accepted that the bound forms of hydroxycinnamic acids are poorly absorbed. However, these phenolic compounds undergo extensive modification during absorption (methylation, sulfation, glucuronidation), therefore the forms reaching the blood and tissues differ from those present in the food and it is challenging to measure the presence all the metabolites and their bioactivities (Pandey & Rizvi, 2009).

1.4.6. Approaches to enhance bioaccessibility of functional ingredients

The most studied approach to enhance the bioaccessibility of functional ingredients is the use of emulsions. Emulsion-based delivery systems, a type of colloidal delivery system, can improve the bioavailability of hydrophobic bioactive ingredients. Compounds are dissolved in carrier oil, which is emulsified with an aqueous emulsifier solution to form an oil-water emulsion and digestion of the carrier oil releases the bioactive compound (McClements & Xiao, 2012). A review by McClements (McClements, 2010) detailed the necessary characteristics of an edible delivery system to improve bioavailability of

lipophilic bioactive ingredients including conjugated linoleic acid (CLA), omega-3 fatty acids and carotenoids. These attributes are:

1. High loading capacity and encapsulation and retention efficiencies
2. Prevention of chemical degradation of chemically labile bioactive compounds e.g. oxidative degradation
3. Compatibility with food/beverage matrix it is to be incorporated into without affecting sensory or quality parameters
4. Resistance to environmental stresses that the food or beverage is exposed to e.g. storage, thermal processing

In addition, the delivery system should be prepared with ingredients generally regarded as safe (GRAS) in a facility practising good manufacturing practices (GMP) and the added benefit of encapsulating the bioactive lipophilic compounds should outweigh the associated costs (McClements, 2010). If multilayer emulsion coatings are used as a delivery system, it is imperative that they are broken down in the gastrointestinal tract to permit release of the bioactive ingredient and any potential health benefits of absorption to occur. Aditya *et al.* (2013) fabricated nanostructured lipid carriers (NLCs) as a potential aid to improve bioaccessibility of genistein and curcumin. These NLCs had 78 – 79 % entrapment efficiency (EE) and 1.2 - 2 % nutraceutical loading for genistein and curcumin, respectively. Encapsulation of genistein and/or curcumin resulted in an increased solubility in simulated intestinal medium (SIM) from < 20 % to > 75 % and \geq 85 % stability in SIM and simulated gastric medium (SGM) for up to 6 hr. Coloaded of genistein and curcumin increased loading efficiency and prostate cancer cell growth inhibition. Nanoemulsions have also been investigated for their potential to increase β -carotene bioaccessibility, using medium-chain (MCT) and/or long-chain triglycerides (LCT) as the lipid carrier and a static *in vitro* digestion procedure. Use of either LCT or MCT nanoemulsions resulted in significantly higher β -carotene bioaccessibility,

compared to nanoemulsions containing LCT-MCT mixtures (Salvia-Trujillo *et al.*, 2013). A similar study has been carried out for quercetin bioaccessibility (Pool *et al.*, 2013). The small particle size may be responsible for the ability of nanoemulsions to increase bioaccessibility of functional ingredients due to increased solubility, greater surface area and entrapment in the mucous layer thus increasing retention time (McClements & Xiao, 2012).

Proteins play a role in the stabilisation of colloidal delivery systems; hydrolysis of these proteins affects their ability to alter droplet charge, droplet aggregation, lipid digestion and bioactive release (Pool *et al.*, 2013). It has been shown that when lycopene is embedded into whey protein matrices, bioavailability is increased to levels of that for tomato paste (Richelle *et al.*, 2002). Other studies also report the use of a protein-stabilised lipid carrier to increase bioaccessibility of quercetin (Pool *et al.*, 2013) and β -carotene (Qian *et al.*, 2012). The study by Qian *et al.* (2012) showed that the presence of β -lactoglobulin inhibited the degradation of β -carotene, compared to the use of Tween 20 to stabilise the nanoemulsion (Qian *et al.*, 2012). The use of colloidal delivery systems is well-established in the area of oral drug delivery, however it is still in its infancy in terms of functional ingredient bioaccessibility. For this reason, more research regarding the development of food-grade colloidal systems to improve bioaccessibility of functional ingredients is needed to provide a comprehensive understanding of their effects *in vitro* and *in vivo*.

1.4.7. Conclusion

This review discussed bioaccessibility assessment and factors affecting bioaccessibility of functional food ingredients, including carotenoids, flavonoids and hydroxycinnamic acids. In addition, potential approaches to improve bioaccessibility were considered. Assessment of the bioaccessibility of functional ingredients is crucial for determining potential

bioactivity. The development of advanced *in vitro* digestion models, particularly dynamic models, provides the opportunity to closely mimic *in vivo* conditions. However, it is necessary to validate and standardise existing *in vitro* procedures for consistency between studies. Elucidating bioaccessibility of functional ingredients is extremely challenging, with a multitude of factors such as food matrix effect, host-related factors and processing affecting the fate of bioactive compounds during digestion. Carotenoids are highly bioaccessible, as are isoflavones, with anthocyanins and bound hydroxycinnamic acids having limited bioaccessibility. To offset this low bioaccessibility, the use of colloidal delivery systems using protein as a stabiliser proves beneficial. Given the increasing interest in bioactive functional ingredients and their incorporation into foods for human consumption optimisation and standardisation of methods to measure bioaccessibility and further research on the development of food-grade colloidal systems are essential.

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1.5. Literature Review Overall Conclusion

The exploitation of BSG as a source of novel bioactive phenolic extracts and protein hydrolysates for inclusion into functional foods represents a potential application to increase the value of and reduce the waste associated with this brewing industry co-product. To date, BSG use has been limited to animal nutrition, however given the potential health benefits associated with both the phenolic and protein components of BSG discussed in this review, its use in human nutrition is favourable.

Phenolic compounds, including hydroxycinnamic acids, have been shown to possess antioxidant, anti-inflammatory, anti-atherogenic and anti-cancer capabilities. Protein hydrolysates from a variety of sources have also demonstrated biological activity, being antioxidative and blood pressure lowering, while also having potential in sports nutrition and the treatment of clinical conditions such as phenylketonuria (PKU) and chronic liver disease. It is expected that extracts prepared from BSG would show similar bioactivities to those discussed in this review.

In addition to evaluating the safety, stability and consumer acceptability of formulated functional food products, clinical trials are essential to validate claims regarding bioactive ingredients. Furthermore, determination of the fate of bioactive ingredients during passage through the gastrointestinal tract should be identified using *in vitro* or *in vivo* gastrointestinal digestion models, to predict bioactive effects *in vivo*.

1.6. Research Objective

The objective of this thesis was to determine the biological activity of phenolic extracts and protein hydrolysates prepared from brewers' spent grain (BSG) and to determine their potential for use as functional food ingredients by measuring bioactivity retention in model food systems.

Antioxidant and immunomodulatory potential of phenolic extracts and protein hydrolysates from BSG was measured using *in vitro* cell culture models. The most bioactive samples were chosen for addition into model food systems and subjected to *in vitro* digestion, before determining the biological activity of the digestates.

Chapters 2, 3 and 4 of this thesis consider phenolic extracts prepared from BSG. The ability of phenolic extracts from BSG to protect against oxidant-induced DNA damage using the comet assay is examined in Chapter 2. Chapter 3 details the hydroxycinnamic acid content of BSG phenolic extracts, determined using high performance liquid chromatography (HPLC). In addition, results of preliminary research regarding the addition of phenolic extracts into foods, namely fruit juices and smoothies, are reported. Chapter 4 studies the antioxidant and anti-inflammatory potential of BSG phenolic extracts, measured using cellular antioxidant activity assays and an enzyme-linked immunosorbent assay (ELISA).

Chapters 5 and 6 examine protein hydrolysates from BSG. Chapter 5 investigates the *in vitro* antioxidant and anti-inflammatory potential of protein samples A - J, by the superoxide dismutase (SOD) and comet assays and ELISA. Using similar methodologies, the bioactivity of protein samples K - Y, including fractionated hydrolysates, is studied in chapter 6.

Chapter 7 details the addition of the most bioactive phenolic extracts and protein hydrolysates from BSG into model food systems. Fortified foods were subjected to an *in*

vitro digestion procedure, followed by assessment of their bioactivity in cell culture, using the comet assay and ELISA.

Chapter 2

Phenolic extracts of Brewers' Spent Grain (BSG) as functional ingredients - assessment of their DNA protective effect against oxidant-induced DNA single strand breaks in U937 cells.

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2.1. Abstract

Brewers' spent grain (BSG), a by-product of the brewing industry, contains high amounts of phenolic acids, which have antioxidant effects. The present study examined the ability of BSG extracts to protect against the genotoxic effects of oxidants, hydrogen peroxide (H_2O_2), 3-Morpholinopyridone hydrochloride (SIN-1), 4-Nitroquinoline 1-oxide (4-NQO) and *tert*-butylhydroperoxide (*t*-BOOH) in U937 cells. Four pale (P1-P4) and four black (B1-B4) BSG extracts were investigated. U937 cells were pre-incubated with BSG extracts, exposed to the oxidants and the DNA damage was measured by the comet assay. The black BSG extracts (B1-B4) significantly protected against H_2O_2 -induced DNA damage. Extract B2, which had the highest phenol content, provided the greatest protection. Extracts P2, B2, B3 and B4 provided significant protection against SIN-1-induced DNA damage. None of the extracts protected against DNA damage induced by *t*-BOOH and 4-NQO. The DNA protective effects of the BSG phenolic extracts may be related to iron chelation.

2.2. Introduction

Brewers' spent grain (BSG) is the residual solid fraction of barley malt remaining after the production of wort for the brewing industry. There is approximately 3.4 million tons of BSG produced annually in the European Union according to Eurostat Data. Currently, BSG is used in animal feed as it is high in protein and fibre (Mussatto *et al.*, 2006) and when combined with nitrogen sources, can provide all the essential amino acids (Huige, 1994). Additional uses for the by-products of the brewing industry would be advantageous due to the increasing cost of their disposal. BSG is a unique source of bioactive ingredients including phenolic acids, particularly the hydroxycinnamic acids, ferulic acid, *p*-coumaric acid, sinapic acid and caffeic acid (Szwajgier *et al.*, 2010).

Plant phenols have been reported to have antioxidant and anti-carcinogenic effects (Hollman, 2001). Flavanoids and hydroxycinnamic acids, which are the predominant polyphenols in BSG, have antioxidant potential and the *in vitro* antioxidant effect of plant polyphenols was shown to be similar to that of the well-known antioxidants, α -tocopherol and ascorbic acid (Rice-Evans *et al.*, 1997). There is a large amount of information available relating to the health effects of foods rich in phenols such as tea (catechins), coffee (chlorogenic acid), wine (resveratrol) and fruit. However, little is known regarding the antioxidant potential of BSG.

Antioxidant activity can be determined by assessing the ability of a compound to protect against oxidant-induced DNA damage (Collins, 2004). Oxidants such as hydrogen peroxide (H_2O_2) and *tert*-butylhydroperoxide (*t*-BOOH) are known to induce DNA damage, such as single strand breaks. It has been shown that polyphenols, such as quercetin and rutin, protect against *t*-BOOH induced DNA damage (Aherne & O'Brien, 2000) and H_2O_2 induced oxidative damage (Musonda & Chipman, 1998). 3-Morpholinopyridone hydrochloride (SIN-1) has been shown to induce DNA damage in Jurkat T cells and 4-Nitroquinoline 1-oxide (4-NQO) induced oxidative DNA damage in HL-60 cells (Abraham *et al.*, 2007; Johnson & Loo, 2000).

The aim of the present study was to assess the ability of phenolic rich BSG extracts to protect against DNA damage in human lymphocytic U937 cells. Four extracts were prepared from both a pale BSG and a black BSG and the total phenolic content (TPC) of the extracts was determined. It was then investigated if the phenolic extracts could inhibit oxidative DNA damage induced by either H_2O_2 , SIN-1, 4-NQO or *t*-BOOH using the alkaline single cell gel electrophoresis (Comet) assay.

2.3. Materials and methods

2.3.1. Materials

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich Ireland Limited, Co. Wicklow, Ireland. U937 cells were obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK).

2.3.2. Sample preparation

Pale BSG was the remaining solid fraction obtained following the removal of wort during the production of beer in the brewing process. Black BSG was the solid fraction remaining following aqueous extraction of barley grains which had been roasted at 200 °C. Both BSG types were stored in 1 kg quantities in polyethylene bags which were vacuum packed and maintained at -20 °C. The test samples designated P1 - P4 and B1 - B4 were obtained from pale (P) and black (B) BSG, respectively. For production of the P1 and B1 samples, 15 g BSG (dw) was first sheared for 20 s at 11,000 rpm using an Ultra Turrax[®] T25 basic high performance disperser (IKA[®] Werke GmbH & Co. KG, Janke and Kunkel-Str.10, D-79219 Staufen, Germany) in 300 mL H₂O. The sheared samples were then allowed to stir gently for 1 hr at room temperature prior to centrifugation. The resulting supernatants were designated P1 and B1. The precipitate obtained from the above process was subjected to two sequential 1 hr extractions in 300 mL 110 mM NaOH at room temperature. The combined supernatants from the above extractions obtained after centrifugation were adjusted to pH 3.8, stirred gently for 15 min at room temperature and then centrifuged. The supernatants obtained were designated as P4 and B4. The precipitates from above were further extracted in the dark with gentle stirring using 200 mL 1 N NaOH at room temperature for 16 hr. The supernatants obtained following centrifugation were designated P2 and B2. The

precipitate was further extracted in the dark for 1 hr with 200 mL H₂O by gently stirring at room temperature and the resultant supernatants obtained following centrifugation were designated P3 and B3 respectively. All centrifugation steps were carried out at 2710 g for 20 min at 10 °C using a Hettich Zentrifugen Universal 320R centrifuge (Andreas Heitich GmbH & Co. KG, Fohrenstr.12, D-78532 Tuttlingen, Germany) and all supernatant samples were adjusted to pH 7.0 using 2 N NaOH or 2 N HCl as required, prior to analysis.

2.3.3. Total phenolic content

The total phenolic content (TPC) of the BSG extracts was determined using the Folin-Ciocalteu method as described previously (Singleton & Rossi, 1965) . The assay is based on the oxidising ability of the Folin-Ciocalteu reagent. A yellow to blue/green colour change is observed and the absorbance is measured spectrophotometrically at 765 nm.

2.3.4. Quantification of ferulic acid content in BSG samples

The reverse-phase HPLC method used was according to Tsao and Yang, 2003. Analysis was carried out using a Waters HPLC system (Waters Corporation, Milford, MA, USA) equipped with a Waters 717 Plus autosampler and a dual absorbance detector. Separation was carried out with a Jupiter 300 C18 column, 5µm particle size (250 x 4.6, L x ID mm, Phenomenex, Cheshire, UK) with a C18 guard column. The mobile phase consisted of 6 % acetic acid in 2 mM sodium acetate (v/v), final pH 2.55 (solvent A) and acetonitrile (solvent B). The flow rate was 1.0 mL per min for a total run time of 70 min and the gradient programme was as follows: 0 % B to 15 % B in 40 min, 15 % B to 30 % B in 15 min, 30 % B to 50 % B in 5 min and 50 % B to 100 % B in 5 min. There was a 70 - 80 min post-run at initial conditions to equilibrate the column. The injection volume was 20

μL and peaks were monitored at 280 nm and 320 nm. All samples were filtered through 0.45 μm filters prior to injection and peak area was related to concentration using a standard curve generated from varying concentrations of ferulic acid.

2.3.4. Ferric Reducing Antioxidant Power (FRAP) Assay

The *in vitro* antioxidant activity of test solutions was estimated according to the procedure of Benzie and Strain (1996) as modified by Griffin and Bhagooli (2004). Solutions of known Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) concentration in the range of 50 - 500 μM were used for calibration and the results from test extracts were expressed as Trolox Equivalents per mL (TE/mL).

2.3.5. Cell culture

U937 cells, human histiocytic lymphoma cells, were maintained in antibiotic-free RPMI-1640 medium supplemented with 10 % (v/v) fetal bovine serum (FBS), at 37 °C in a 5 % CO_2 atmosphere. Cells were adjusted to a density of 1×10^5 cells/mL, in reduced serum media (2.5 % FBS) for comet experiments.

2.3.6. Preparation of oxidants

Each of the oxidants (H_2O_2 , SIN-1, 4-NQO and *t*-BOOH) was dissolved in distilled, deionised water. A 20 mM stock solution of H_2O_2 was prepared and added to 2 mL cells to give final concentrations ranging from 50 - 200 μM . For SIN-1, a 0.1 M stock solution was prepared and concentrations of 1, 2 and 4 mM were added to cells. The stock solution of 4-NQO was prepared to a concentration of 200 $\mu\text{g/mL}$ and was added to cells at final concentrations of 2, 4 and 6 $\mu\text{g/mL}$. A stock solution (40 mM) of *t*-BOOH was prepared and added to cells at final concentrations of 200, 400 and 800 μM .

2.3.7. Comet assay

U937 cells (2 mL) were seeded in a 6-well plate at a density of 1×10^5 cells/mL in RPMI-1640 medium supplemented with 2.5 % FBS. Initial cytotoxicity testing with the (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt (MTT) assay indicated the concentration of BSG phenolic extracts and ferulic acid standard to use for assay, 2.5 % (v/v) and 1 μ g/mL respectively. Cells were incubated for 24 hr with either 2.5 % (v/v) (50 μ L in 2 mL cells) phenolic extracts from the BSG or 1 μ g/mL ferulic acid. Cells were subsequently treated with H₂O₂ (50 μ M for 30 min), SIN-1 (4 mM for 90 min), 4-NQO (2 μ g/mL for 90 min) or *t*-BOOH (400 μ M for 60 min). Following exposure of the cells to the various treatments, cell viability was measured using the fluorescein diacetate/ethidium bromide (FDA/EtBr) assay which stains live cells green and dead cells red. The FDA/EtBr assay was conducted simultaneously to each comet assay to ensure cell viability did not decrease below 90 %, which could invalidate the results from the comet assay. Oxidative DNA damage to the U937 cells was assessed using the comet assay adapted from Tice *et al.* (1991). Firstly, slides were prepared by coating with 1 % (w/v) normal gelling agarose (NGA). Cells (30 μ L) were then suspended in 70 μ L of 1 % (w/v) low melting point agarose (LMP), covered with a coverslip and placed on ice to solidify. Slides were submerged in the comet lysing solution at 4 °C for 1 hr, before being placed in the comet electrophoresis solution for 40 min. Electrophoresis was carried out at 21 V, 300 mA for 25 min. Neutralisation of the slides was performed by washing three times with 0.4 M Tris for 5 min. Slides were rinsed with distilled H₂O and 20 μ g/mL EtBr was placed on the slides for 5 min. Following a final rinse with H₂O, coverslips were replaced and 50 cells were scored for each slide using a fluorescence microscope (Optiphot-2, Nikon) at wavelength 510 - 550nm and Komet 5.5 image analysis software.

2.3.8. Statistical Analysis

All data points are the mean and standard error values of at least three independent experiments. Data were analysed by ANOVA followed by Dunnett's test. The software employed for statistical analysis was GraphPad Prism, version 4.00 for windows (GraphPad software, San Diego, California, USA).

2.4. Results and discussion

2.4.1. Total Phenolic Content of BSG extracts

Previous studies have extracted phenolic acids from BSG using methods based on acidic hydrolysis or saponification. Saponification involves the treatment of samples with 1 - 4 M NaOH solution, similar to the method used in the present study, and has previously been used to extract hydroxycinnamic acids from BSG (Bartolome *et al.*, 2002). Four extracts were prepared from both the pale BSG (P1 - P4) and the black BSG (B1 - B4) as outlined in Section 2.3.2.. The total phenolic content of each of the phenolic extracts from BSG was measured (Table 1). Extracts P1 and B1 contain the water soluble or free phenolics extracted from BSG and the lower TPC of these samples demonstrates that there is a low concentration of free phenolics in BSG. The second phase of the extraction procedure released the bound phenolics as represented by P2 and B2. P2 and B2 have the highest TPC (Table 1) suggesting that it is predominantly bound phenolics that are present in BSG. Extracts P3 and B3 were released at the third step of the extraction and contain the remaining bound phenolics from the BSG sample (Table 1). The phenolic fractions collected at the final phase of the extraction procedure, P4 and B4, had a lower TPC than the two previous phenolic extracts (P2, B2 and P3, B3) obtained using 1M NaOH. It has previously been reported that the majority of the phenolic acids in BSG are in the bound form (Forssell *et al.*, 2008). It has also been found that the bound

Table 1: Total phenolic content (TPC) of phenolic extracts from Brewer's Spent Grain (BSG).

BSG Extract ^a	TPC (mg gallic acid equivalents (GAE)/mL)
P1	0.014 ± 0.000 ^b (0.35)
P2	0.641 ± 0.024 ^b (16.03)
P3	0.225 ± 0.003 ^b (5.63)
P4	0.059 ± 0.001 ^b (1.48)
B1	0.083 ± 0.001 ^b (2.08)
B2	0.732 ± 0.020 ^b (18.30)
B3	0.267 ± 0.010 ^b (6.68)
B4	0.128 ± 0.010 ^b (3.20)

^aP1 - P4 represent the four pale BSG extracts while B1 - B4 represent the four black BSG extracts. ^b Values are mean ± SE of three independent experiments. Statistical analysis was ANOVA followed by Dunnett's test. Data in brackets represent the total phenolic content (µg GAE/mL) of 2.5 % (v/v) extract (used for analysis).

phenolics in barley, which is used in the brewing process, are predominantly ferulic and *p*-coumaric acid (Holtekjølen *et al.*, 2006). Reverse-phase HPLC was used to quantify ferulic acid, the principle phenolic acid found in BSG, according to the procedure of Tsao and Yang (2003). Ferulic acid could not be detected in the aqueous extracts, P1 and B1. Higher levels of ferulic acid were detected in the pale as compared to the black extracts. The values obtained for the pale extracts were P2, $113.96 \pm 0.88 \mu\text{g/mL}$; P3, $33.81 \pm 2.42 \mu\text{g/mL}$ and P4, $17.59 \pm 0.40 \mu\text{g/mL}$ while those for the black extracts were B2, $27.31 \pm 0.69 \mu\text{g/mL}$; B3, $10.70 \pm 0.28 \mu\text{g/mL}$ and B4, $5.48 \pm 0.06 \mu\text{g/mL}$. A more detailed characterisation of other phenolics in the BSG samples is ongoing.

2.4.2. Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay measures the ability of a compound to reduce Fe^{3+} (ferric ion) to Fe^{2+} (ferrous ion), thus indicating the antioxidant potential of a compound (Benzie & Strain, 1996). Extracts B2, P2, B3 and P3 had the highest FRAP activity and the black extracts showed best antioxidant potential (Table 2). The FRAP activity correlates with the total phenolic content of the BSG extracts (Table 1); extracts with highest TPC showing greatest antioxidant effects. Numerous studies have reported a relationship between TPC and FRAP activity, with higher TPC resulting in higher ferric reducing antioxidant power (Pantelidis *et al.*, 2007; Chu *et al.*, 2002).

2.4.3. Ability of BSG extracts to protect against oxidant-induced DNA damage

The optimal conditions for the induction of maximal DNA damage without affecting cell viability was determined for each of the oxidants in U937 cells. H_2O_2 , at $50 \mu\text{M}$ for 30 min, was found to induce DNA damage with approximately 40 % tail DNA. *t*-BOOH was not as genotoxic as H_2O_2 and $400 \mu\text{M}$ *t*-BOOH for 60 min resulted in 15.5 % tail

Table 2: Antioxidant activity of phenolic BSG extracts, as measured using the Ferric Reducing Antioxidant Potential (FRAP) assay.

BSG Extract ^a	FRAP (μM trolox equivalents (TE)/mL)
P1	0.01 ± 0.00
P2	0.36 ± 0.01
P3	0.13 ± 0.00
P4	0.06 ± 0.00
B1	0.06 ± 0.00
B2	0.53 ± 0.02
B3	0.17 ± 0.00
B4	0.10 ± 0.01

^aP1 - P4 represent the four pale BSG extracts while B1 - B4 represent the four black BSG extracts. ^b Values are mean \pm SD of three independent experiments. Statistical analysis was ANOVA followed by Dunnett's test.

DNA. The selected concentrations of H_2O_2 and *t*-BOOH are similar to those previously employed by Aherne and O'Brien (2000). Few studies have examined the genotoxic effects of SIN-1 and 4-NQO in U937 cells, as assessed using the comet assay, therefore a dose response study for these compounds was carried out. Increasing concentrations of SIN-1 caused a corresponding increase in the percent tail DNA to a maximum of 37.3 % at 4 mM SIN-1 following 90 min incubation (Table 3). 4-NQO was incubated for 90 min at concentrations ranging from 0 - 8 μ g/mL and a concentration of 2 μ g/mL was used to determine the protective effects of the BSG extracts (Table 3). Each of the oxidants ultimately result in the formation of single strand breaks in the DNA, however the mechanisms involved differ. H_2O_2 produces hydroxyl radicals (OH^\bullet) by means of the Fenton reaction in an iron dependent reaction. OH^\bullet radicals cause single strand breaks by attacking the ribose on the DNA backbone (Benhusein *et al.*, 2010). SIN-1 produces nitric oxide (NO) and superoxide anion (O_2^-), which subsequently form peroxynitrite ($ONOO^-$). O_2^- is converted to H_2O_2 which results in DNA damage, by a Fenton type reaction. Peroxynitrite can also generate hydroxyl radical in an iron-independent manner (Inoue & Kawanishi, 1995). 4-NQO mimics the action of UV and previous studies have shown that cell treatment with 4-NQO can lead to the production of H_2O_2 , superoxide and hydroxyl radicals and a substantial amount of oxidised DNA bases (Arima *et al.*, 2006). It has been suggested that Cu^{2+} has an essential role in DNA damage induced by 4-NQO, whereas iron does not (Yamamoto *et al.*, 1993). *t*-BOOH causes lipid peroxidation and triggers the Ca^{2+} -dependent formation of H_2O_2 which results in DNA cleavage. The production of additional DNA damaging species by *t*-BOOH results in DNA lesions comparable to those produced by the hydroxyl radical. DNA single-strand breakage induced by *t*-BOOH has been shown to occur in an iron dependent manner, however studies have also demonstrated that *t*-BOOH-induced DNA damage is less dependent on iron than H_2O_2 -induced DNA damage (Kruszewski *et al.*, 2008).

Table 3: Dose response effects of BSG extracts on oxidative DNA damage induced by 3-Morpholinosydnonimine hydrochloride (SIN-1) or 4-Nitroquinoline 1-oxide (4-NQO) following 90 min incubation, as measured using the Comet assay.

	DNA damage (% Tail DNA)
Control	3.3 ± 0.4 ^a
1 mM SIN-1	4.8 ± 0.6 ^a
2 mM SIN-1	12.0 ± 0.7 ^{a *}
4 mM SIN-1	37.3 ± 1.2 ^{a *}
2 µg/mL 4-NQO	41.2 ± 4.5 ^{a *}
4 µg/mL 4-NQO	51.2 ± 6.7 ^{a *}
6 µg/mL 4-NQO	47.9 ± 6.7 ^{a *}
8 µg/mL 4-NQO	46.3 ± 3.2 ^{a *}

^a Values are mean ± SE of three independent experiments. Statistical analysis was by ANOVA followed by Dunnett's test. * Denotes a significant difference (P < 0.05) in DNA damage, relative to control.

To assess the DNA protective effects of the phenolic extracts of pale and black BSG, U937 cells were pretreated with the extracts for 24 hr prior to exposing the cells to the oxidants. The use of 2.5 % BSG extracts or 1 $\mu\text{g}/\text{mL}$ ferulic acid did not affect cell viability, with viability $> 90\%$ for all conditions used. Both ferulic acid and the BSG extracts alone did not induce DNA damage at the concentrations used in the present study (data not shown). The addition of 50 μM H_2O_2 to U937 cells for 30 min significantly ($P < 0.05$) increased tail DNA to approximately 40 % (Table 4). The black BSG extracts and ferulic acid significantly ($P < 0.05$) reduced the percentage tail DNA in H_2O_2 treated U937 cells (Table 4). The black BSG extracts have a higher TPC than the corresponding pale BSG extracts, thus, a relationship between the TPC of the BSG extracts and protection against H_2O_2 induced DNA damage is evident. Ferulic acid, *p*-coumaric acid, sinapic acid and caffeic acid are the phenolic acids present at highest concentrations in BSG (Szwajgier *et al.*, 2010). Previous studies have demonstrated that pure phenolic compounds, ferulic acid and caffeic acid protect against DNA damage caused by H_2O_2 , as measured by the comet assay (Wang *et al.*, 2008; Nousis *et al.*, 2005). In a study conducted by Wang *et al.* (2008b) feruloyl oligosaccharides (FOs), the ferulic acid ester of oligosaccharides, from wheat bran, protected against H_2O_2 -induced DNA damage in lymphocytes, as measured by the comet assay. The authors attributed this protection to the antioxidant capacity of the ferulic acid moiety. Olive oil phenolics have also shown a protective effect against DNA damage induced by H_2O_2 , as assessed by the comet assay (Quiles *et al.*, 2002).

The exposure of U937 cells to SIN-1 (4 mM) for 90 min resulted in an increase in tail DNA from a control, untreated level of 4.3 % to 40 % (Table 5). The BSG extracts which significantly ($P < 0.05$) protected against SIN-1-induced DNA damage were pale BSG extract P2, which is the pale sample with the highest polyphenol content and black BSG extracts B2, B3 and B4 (Table 5). Extract B2 reduced the level of DNA damage to

Table 4: DNA damage in U937 cells treated with 50 μM H_2O_2 for 30 min following 24 h incubation with either 2.5 % (v/v) pale (P1 - P4) and black (B1 - B4) brewers' spent grain extracts or ferulic acid (FA, 1 $\mu\text{g}/\text{mL}$).

Test	DNA damage	Test	DNA damage
Sample	(% Tail DNA)	Sample	(% Tail DNA)
Control	3.2 ± 0.5^a	Control	2.6 ± 0.3^a
H_2O_2	40.3 ± 0.3^a	H_2O_2	42.1 ± 2.7^a
P1	30.3 ± 7.3^a	B1	$25.8 \pm 7.5^{a*}$
P2	29.0 ± 6.0^a	B2	$9.6 \pm 1.1^{a*}$
P3	29.9 ± 3.9^a	B3	$16.0 \pm 2.0^{a*}$
P4	23.1 ± 3.2^a	B4	$14.0 \pm 3.8^{a*}$
FA	$20.3 \pm 5.7^{a*}$	FA	$17.1 \pm 0.8^{a*}$

^a Values are mean \pm SE of three independent experiments. Statistical analysis was by ANOVA followed by Dunnett's test. * Denotes a significant difference ($P < 0.05$) in DNA damage, relative to H_2O_2 control.

Table 5: DNA damage in U937 cells treated with 4 mM 3-morpholinosydnonimine hydrochloride (SIN-1) for 90 min following 24 hr incubation with either 2.5 % (v/v) pale (P1 - P4) and black (B1 - B4) brewers' spent grain extracts or ferulic acid (FA, 1 µg/mL).

Test	DNA damage	Test	DNA damage
Sample	(% Tail DNA)	Sample	(% Tail DNA)
Control	4.3 ± 0.4 ^a	Control	4.3 ± 0.4 ^a
SIN-1	40.0 ± 3.9 ^a	SIN-1	40.0 ± 3.9 ^a
P1	32.8 ± 4.9 ^a	B1	33.6 ± 2.9 ^a
P2	22.7 ± 1.9 ^{a*}	B2	6.9 ± 1.0 ^{a*}
P3	33.8 ± 2.9 ^a	B3	18.1 ± 0.3 ^{a*}
P4	32.2 ± 6.1 ^a	B4	24.1 ± 2.2 ^{a*}
FA	30.8 ± 3.0 ^a	FA	30.8 ± 3.0 ^a

^a Values are mean ± SE of three independent experiments. Statistical analysis was by ANOVA followed by Dunnett's test. * Denotes a significant difference (P < 0.05) in DNA damage, relative to SIN-1 control.

6.9 %, which is almost a return to control level (4.3 %). Ferulic acid did not significantly ($P < 0.05$) protect against SIN-1 induced DNA damage. Similar to H₂O₂-induced DNA damage, there appeared to be a relationship between the TPC of the extracts and the level of protection afforded against SIN-1-induced DNA damage. Sinapic acid, the third most concentrated phenolic acid in BSG (Szwajgier *et al.*, 2010) has been shown to scavenge peroxynitrite (ONOO⁻) induced by SIN-1 (Zou *et al.*, 2002). Similarly, Johnson and Loo (2000) reported that two phenolic compounds, epigallocatechin gallate and quercetin, scavenged the free radicals produced by H₂O₂ and SIN-1 in Jurkat cells.

Incubation of U937 cells with 4-NQO (2 µg/mL) resulted in a significant increase in tail DNA to 39.5 % (Table 6). Neither the BSG extracts nor the pure ferulic acid significantly protected against the DNA damage induced by 4-NQO (Table 6). Abraham *et al.* (2007) found that the polyphenol, chlorogenic acid, did not significantly reduce 4-NQO induced DNA damage. Chlorogenic acid is present at small concentrations in BSG (Szwajgier *et al.*, 2010). Kuroda (1996) also reported that the simultaneous treatment of V79 cells with green tea polyphenols (catechins) and 4-NQO did not inhibit mutagenicity induced by 4-NQO.

The incubation of U937 cells with 400 µM *t*-BOOH for 60 min significantly ($P < 0.05$) increased the percent tail DNA to 15.5 % (Table 7). None of the phenolic BSG extracts significantly protected against *t*-BOOH induced DNA damage. These results are in contrast with previously reported data which found that plant-derived phenolic compounds, including quercetin, protected against DNA damage induced by *t*-BOOH in the U937 cell line (Sestili *et al.*, 2002). It has been shown that caffeic acid protected against oxidative stress induced by *t*-BOOH by inhibiting lipid peroxidation (Nardini *et al.*, 1998). Phenolic extracts from swallow root also protected against *t*-BOOH induced DNA damage (Harish Nayaka *et al.*, 2010), and it was suggested that *p*-coumaric acid

Table 6: DNA damage in U937 cells treated with 2 µg/mL 4-nitroquinoline 1-oxide (4-NQO) for 90 min following 24 hr incubation with either 2.5 % (v/v) pale (P1 - P4) and black (B1 - B4) brewers' spent grain extracts or ferulic acid (FA, 1 µg/mL).

Test	DNA damage	Test	DNA damage
Sample	(% Tail DNA)	Sample	(% Tail DNA)
Control	3.1 ± 0.3 ^a	Control	3.1 ± 0.3 ^a
4-NQO	39.5 ± 1.1 ^a	4-NQO	39.5 ± 1.1 ^a
P1	39.0 ± 6.8 ^a	B1	36.1 ± 5.7 ^a
P2	40.7 ± 4.3 ^a	B2	31.9 ± 3.2 ^a
P3	37.3 ± 3.2 ^a	B3	33.4 ± 2.4 ^a
P4	37.8 ± 3.6 ^a	B4	33.7 ± 6.8 ^a
FA	32.6 ± 5.2 ^a	FA	32.6 ± 5.2 ^a

^a Values are mean ± SE of three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test was employed to compare 4-NQO-treated cells with those preincubated with BSG extract prior to 4-NQO exposure.

Table 7: DNA damage in U937 cells treated with 400 μ M *tert*-butylhydroperoxide (*t*-BOOH) for 60 min following 24 h incubation with either 2.5 % (v/v) pale (P1-P4) and black (B1-B4) brewers' spent grain extracts or ferulic acid (FA, 1 μ g/mL).

Test	DNA damage	Test	DNA damage
Sample	(% Tail DNA)	Sample	(% Tail DNA)
Control	4.7 \pm 0.6 ^a	Control	4.7 \pm 0.6 ^a
<i>t</i> -BOOH	15.5 \pm 0.8 ^a	<i>t</i> -BOOH	15.5 \pm 0.8 ^a
P1	14.5 \pm 0.8 ^a	B1	13.6 \pm 0.9 ^a
P2	22.6 \pm 3.0 ^a	B2	18.5 \pm 3.3 ^a
P3	22.1 \pm 3.1 ^a	B3	16.6 \pm 1.8 ^a
P4	17.4 \pm 2.6 ^a	B4	14.0 \pm 2.7 ^a
FA	11.0 \pm 1.9 ^a	FA	11.0 \pm 1.9 ^a

^a Values are mean \pm SE of four independent experiments. Statistical analysis by ANOVA followed by Dunnett's test was employed to compare *t*-BOOH treated cells with those preincubated with BSG extract prior to *t*-BOOH exposure.

had a major role in this antioxidant effect and that the bound phenolics were more effective than free phenolics.

The phenolic extracts of BSG protected against the genotoxic effects of H₂O₂ and SIN-1 but not 4-NQO and *t*-BOOH. DNA damage induced by H₂O₂ is more dependent on iron than DNA damage induced by *t*-BOOH (Kruszewski *et al.*, 2008). Polyphenols act as metal chelators and bind Cu and iron (Lopes *et al.*, 1999) and this may be one of the mechanisms dictating the genoprotective effects of the phenolic extracts of the BSG. Studies carried out by Perron *et al.* (2008, 2011) demonstrated that selected pure polyphenols protected more against the DNA damaging effects of iron than the DNA damaging effects of copper, as assessed in plasmid DNA purified from *E. coli*, using a gel electrophoresis method. In addition, results of the FRAP assay support the suggestion that the BSG extracts protect against oxidant-induced DNA damage by iron chelation. These extracts, particularly the black BSG extracts, showed the ability to reduce the ferric oxidant Fe³⁺ to Fe²⁺, thus reducing reactive species. The BSG extracts may also act by scavenging reactive oxygen species and reducing the resultant DNA damage as has previously been demonstrated for pure polyphenols (Bellion *et al.*, 2010). The protective effects of the BSG extracts correlated with their total phenolic content for both pale and black BSG extracts. Black BSG extracts were more protective at comparable TPC.

The black phenolic extracts were found to contain lower ferulic acid levels than the pale phenolic extracts, as detailed previously. Since the black phenolic extracts give greatest protection against DNA damage induced by H₂O₂ and SIN-1, it can be deduced that ferulic acid is not the principle antioxidant entity in the BSG phenolic extracts. This is further evidenced by the fact that B2, B3 and B4 (samples with highest TPC, but lower in ferulic acid than pale extracts) significantly protected against SIN-1 induced DNA damage, whereas the pale extracts P1, P3, P4 and ferulic acid standard did not (Table 5).

Preliminary evidence indicates that high molecular weight phenolic components in the black BSG samples may be the main contributors to the observed antioxidant effects.

In conclusion, the phenolic extracts of BSG protected against the genotoxic effects of H₂O₂ and SIN-1, possibly by chelating iron and or scavenging reactive oxygen species (ROS). The BSG phenolic extracts with highest TPC had highest FRAP activity and gave greatest protection against oxidant-induced DNA damage. BSG may have the potential to be developed as a food additive or dietary supplement with possible health promoting properties.

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Chapter 3

A study of the hydroxycinnamic acid content of barley and brewers' spent grain (BSG) and the potential to incorporate phenolic extracts of BSG as antioxidants into fruit beverages.

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3.1. Abstract

The hydroxycinnamic acid (HA) content of starting barley for brewers' spent grains (BSG), whole BSG and phenolic extracts from BSG was measured using high performance liquid chromatography (HPLC) and correlated with *in vitro* antioxidant potential. The effect of BSG phenolic extracts on antioxidant activity of fruit beverages was also assessed (using the total phenolic content (TPC), 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays). The concentration of HA present in barley extract and BSG was in the order of ferulic acid (FA), *p*-coumaric acid (*p*-CA) derivatives, FA derivatives, *p*-CA, caffeic acid (CA) and CA derivatives. Results suggested that brewing and roasting decreased the HA content. Antioxidant activity was significantly ($P < 0.05$) correlated with caffeic acid ($R^2 = 0.8309$) and total HA ($R^2 = 0.3942$) concentrations. Addition of extracts to fruit beverages resulted in a significant ($P < 0.05$) increase in antioxidant activity of cranberry juice, measured by the FRAP assay. *In vitro* digestion significantly ($P < 0.05$) reduced TPC, DPPH and FRAP activity of the fruit beverages.

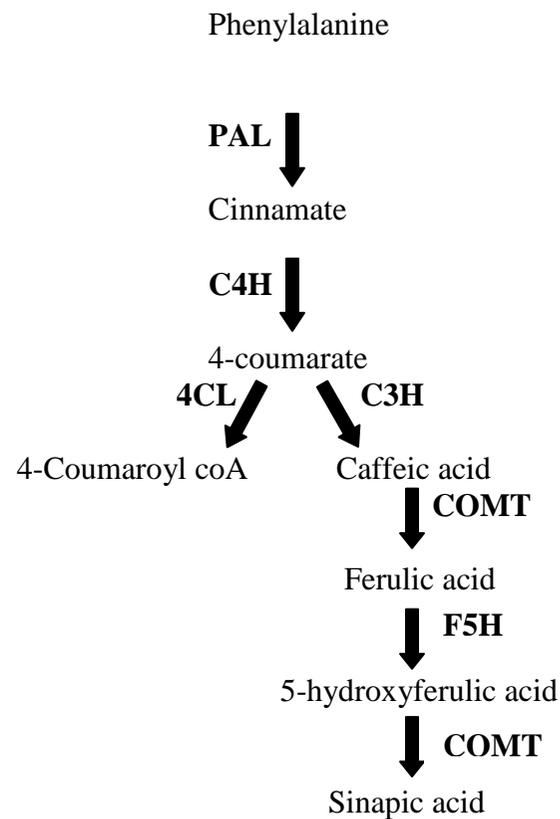
3.2. Introduction

Brewers' spent grain (BSG) is the solid fraction that remains following wort production, the first step of the brewing process. The barley used for brewing can be either pale (unroasted) or black (roasted to approximately 200 °C prior to use), giving rise to pale and black BSG, respectively. BSG is a low-value co-product of the brewing industry (Mussatto *et al.*, 2006). According to Eurostat Data, approximately 3.4 million tons of BSG are produced annually in the E.U.. Due to its high moisture content (75 - 80 %), wet BSG is a perishable product that is difficult to market, therefore some breweries regard BSG as a waste rather than a co-product (Valverde, 1994). BSG contains approximately 20 % protein, 70 % fibre and 1.2 % mono- and di-meric phenolic acids (Mussatto *et al.*,

2006) and hence has a high nutritional value. Currently, BSG is used as a feed supplement for animals, particularly dairy cattle, however, due to its favourable nutritional composition (Mussatto *et al.*, 2006), BSG has the potential to be exploited for human nutrition. Barley is beneficial to human health as a source of both fibre and phenolic acids and has been reported to reduce plasma levels of low-density lipoprotein (LDL) cholesterol and triacylglycerol and also to increase stool volume in healthy women (Li *et al.*, 2003). It is possible that BSG may confer health benefits similar to those exhibited by barley which would create opportunities to reduce BSG waste, by exploiting the nutritional and health protective potential of this co-product (Mussatto *et al.*, 2006).

Polyphenols are the most abundant antioxidants in the human diet and they may support the inherent cellular antioxidant defence system which can become overwhelmed during periods of oxidative stress (Sander *et al.*, 2004). The antioxidant properties of barley, beer and BSG, measured by the oxidation of methyl linoleate, low density lipoprotein (LDL) oxidation and the comet assay, respectively, have been related to their total phenolic content (Maillard *et al.*, 1996; Vinson *et al.*, 2003; McCarthy *et al.*, 2012). Hydroxycinnamic acids are hydroxy derivatives of cinnamic acid, produced by the phenylpropanoid pathway (Figure 1a) and are the primary class of phenolic compounds present in barley, beer and BSG. Hydroxycinnamic acids have a C6 - C3 skeleton and differences in the substituents of the aromatic ring yield different phenolic acids including *p*-coumaric, caffeic, ferulic and sinapic acid (Figure 1b). Ferulic and *p*-coumaric acids are present in barley (Maillard *et al.*, 1996) and beer, which also contains syringic, caffeic and vanillic acids (Vinson *et al.*, 2003). Hydroxycinnamic acids have demonstrated antioxidant effects (Kikuzaki *et al.*, 2002). A previous study has shown that phenolic extracts prepared from BSG have antioxidant activity and the BSG extracts prepared from barley which was roasted prior to brewing (black BSG) demonstrated the highest antioxidant potential (McCarthy *et al.*, 2012).

(a)



(b)

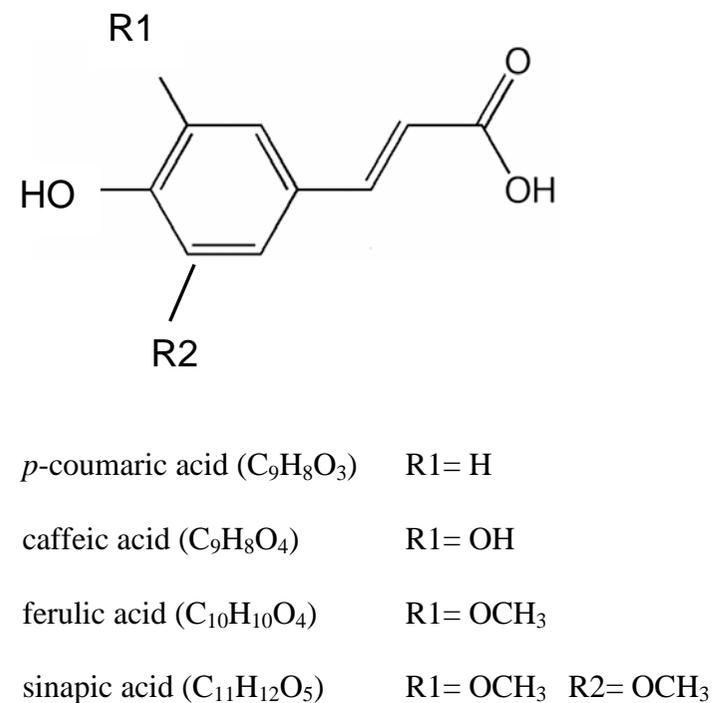


Figure 1: (a) Biosynthetic pathway of hydroxycinnamic acids, the phenylpropanoid pathway. PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate-CoA ligase; C3H, coumaroyl-quininate/shikimate 3-hydroxylase; COMT, caffeic acid: 5-hydroxyferulic acid O-methyltransferase; F5H, ferulate-5-hydroxylase. (b) The general C₆ - C₃ skeleton structure of hydroxycinnamic acids, and the side chains and molecular formula for common hydroxycinnamic acids.

There is a consumer demand for plant-based functional foods that have the potential to improve health and well-being. As a consequence functional fruit juices and drinks have been developed in a number of countries including Poland, New Zealand and the U.S.A. (Sun-Waterhouse, 2011).

Whole BSG has been added to a range of foods including cookies (Öztürk *et al.*, 2002), bread (Stojceska & Ainsworth, 2008) and frankfurters (Özvural *et al.*, 2009). The addition of BSG to these foods increased the protein and fibre content and consumer acceptability was not affected. However, none of these studies investigated the effect of BSG extract supplementation on the phenolic content of the foods.

The stability of polyphenols during digestion and absorption is dependent on the compound ingested and the food matrix. Polyphenols from Concord grape juice were stable during gastric digestion (pH 2) but significant losses were observed in several of the polyphenols during the duodenal phase (pH 7) (Stalmach *et al.*, 2012). Hydroxycinnamic acids were found to be relatively stable during the digestion process in comparison with anthocyanidins, procyanidins and flavonols.

The aim of the present study was to characterise and quantify the most abundant hydroxycinnamic acids in whole ground barley grain, pale BSG, black BSG and BSG phenolic extracts, using high performance liquid chromatography (HPLC), in order to determine the potential of BSG phenolic extracts to be exploited for use in functional foods. The effect of BSG phenolic extracts on antioxidant activity of fruit juices and smoothies was also investigated to give an indication of their potential for use as functional food ingredients.

3.3. Materials and Methods

3.3.1. Materials

Sodium acetate trihydrate, iron sulphate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) and iron chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) were purchased from BDH Laboratory Supplies, Dorset, England. Hydrochloric acid and methanol were purchased from Roth (Karlsruhe, Germany), acetic acid from Merck (Darmstadt, Germany) and acetonitrile from J. T. Baker (Grießheim, Germany). All chemicals and solvents were HPLC grade. All other chemicals unless otherwise stated were purchased from Sigma-Aldrich Ireland Limited, Co. Wicklow, Ireland.

3.3.2. Barley, whole brewers' spent grain (BSG) and BSG phenolic extracts

Barley samples (saffron variety) and wet whole BSG samples were obtained from a local brewery. Wet BSG samples were maintained at $-20\text{ }^\circ\text{C}$ until required for preparation. To prepare, all samples were oven-dried at $60\text{ }^\circ\text{C}$ for 18 hr, and subsequently fine-milled using a Micromark Minigrinder (Supplier: Des Kavanagh Electrical, Galway, Ireland), pulsed for 1 min duration. Preparation of the phenolic extracts from pale and black BSG has been described previously (McCarthy *et al.*, 2012). Samples, designated P1 - P4 and B1 - B4 were acquired from pale and black BSG, respectively. Briefly, P1 and B1 were the supernatants obtained following shearing in water and centrifugation. P2 and B2 were extracted with 110 mM NaOH and subsequently 1 N NaOH. P3 and B3 are aqueous washes of sediments obtained during preparation of P2 and B2 extracts. The supernatants remaining following acid precipitation at pH 3.8 and centrifugation were designated P4 and B4. Whole ground barley and whole ground dried BSG are referred to as barley and BSG throughout.

3.3.3. Sample preparation for HPLC-DAD-MS analysis

Hydroxycinnamic acids were extracted from barley and BSG samples using a modification of the method previously described (Schütz *et al.*, 2004). To analyze the hydroxycinnamic acids, sample (2 g) was extracted in duplicate using 60 % aqueous methanol on a magnetic stirrer plate for 1.5 hr. The extract was passed through a fluted filter and subsequently evaporated to dryness. The residue was dissolved in 5 mL distilled water and then passed through a cellulose mixed ether-membrane filter (CME filter) prior to HPLC analysis coupled with diode array detection (DAD) and mass spectrometry (MS).

3.3.4. Analysis of barley, BSG and BSG phenolic extracts - HPLC-DAD-MS

The hydroxycinnamic acid derivatives were quantified using a method described previously (Neugart *et al.*, 2012). An HPLC series 1100 by Agilent (Waldbronn, Germany) consisting of a degasser, binary pump, autosampler, column oven and photodiode array detector was used. Extracts were separated at 30 °C on a Phenomenex Prodigy column (ODS, 150 X 3.0 mm, 5 µm, 100 Å) using a water/acetonitrile gradient. Solvent A consisted of 99.5 % water and 0.5 % acetic acid; solvent B was 100 % acetonitrile. The following gradient was used: 5 - 7 % (0 - 12 min), 7 - 9 % (12 - 25 min), 9 - 12 % (25 - 45 min), 12 - 15 % (45 - 100 min), 15 % isocratic (100 - 150 min), 15 - 50 % (150 - 155 min), 50 % isocratic (155 - 165 min), 50 - 5 % isocratic (165 - 170 min) and 5 % isocratic (170 - 175 min). The flow rate used was 0.4 mLmin⁻¹ and the detector wavelength was 320 nm. The hydroxycinnamic acid derivatives were identified as deprotonated molecular ions and mass fragment ions by HPLC-DAD/ESI-MSⁿ using an Agilent series 1100 ion trap mass spectrometer in negative ionization mode. Nitrogen was used as the drying gas (12 L min⁻¹, 350 °C) in addition to nebulizer gas (40 psi). Helium was used as the collision gas in the ion trap. The standards, ferulic acid and caffeic acid

(Roth, Karlsruhe, Germany), were used for external calibration curves at concentrations ranging from 0.1 to 10 mg/100 mL. *p*-Coumaric, sinapic and unknown acid concentrations were calculated as ferulic acid equivalents.

3.3.5. Analysis of barley, BSG and BSG phenolic extracts - total phenolic content (TPC)

For determination of the TPC of barley and BSG an extraction step was necessary, BSG phenolic extracts were analysed directly. Extraction was carried out as previously described (Sharma & Gujral, 2010). Acidified methanol (HCl/methanol/water, 1:80:10 v/v/v) was added to 200 mg sample and incubated at room temperature (25 °C) for 2 hr. The supernatant was isolated following centrifugation at 3000 g and all experiments were carried out within 24 hr. The Folin-Ciocalteu method as described previously (Singleton & Rossi, 1965) was used to measure the antioxidant capacity of the barley, BSG and BSG phenolic extracts (P1 - P4; B1 - B4). This assay is based on the ability of a compound to reduce the Folin-Ciocalteu reagent (FCR), resulting in a yellow to blue/green colour change that is measured spectrophotometrically at 765 nm. The TPC assay is non-specific to phenols and measures any reducing agent. Results were expressed as milligram gallic acid equivalents per gram dry weight (g_{dw}) barley/whole BSG (mg GAE/ g_{dw}) and milligram gallic acid equivalents per mL phenolic extract (mg GAE/mL).

3.3.6. Analysis of barley, BSG and BSG phenolic extracts - 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) assay

Again it was necessary to perform an extraction procedure for barley and BSG samples before determining the DPPH radical scavenging potential of the extracts. For extraction, 100 mg barley or BSG was added to 1 mL methanol for 2 hr at room temperature (25 °C), followed by centrifugation for 10 min at 3000 g (Sharma & Gujral, 2010). The supernatant was used for subsequent analysis and all experiments were carried out within

24 hr of extraction. The DPPH assay was carried out according to the method described previously (Brand-Williams *et al.*, 1995). Known concentrations of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), ranging from 0.04 to 0.40 μM were used to prepare a standard curve for calibration. To account for background colour in the samples, colour blanks were prepared with 100 μL sample and 3.9 mL methanol (MetOH) where necessary. Absorbance (at 515 nm) of all samples was determined following a 30 min incubation with the DPPH reagent (0.0238 $\mu\text{g/mL}$). This assay gives a measure of antioxidant potential, based on the ability of a compound to scavenge the DPPH radical by hydrogen donation. Results were expressed as % DPPH inhibition:

$$\% \text{ DPPH inhibition} = [(\text{Abs}_{\text{blank}} - \text{Abs}_{\text{sample}})/\text{Abs}_{\text{blank}}] \times 100$$

3.3.7. Preparation of fruit beverages

A total of four commercially available fruit juices or smoothies were purchased from a local supermarket; Sungrown Pure White Grape Juice (Tesco, UK), OceanSpray Cranberry Classic Juice Drink (OceanSpray Cranberries Inc., MA, USA), Innocent Strawberry and Banana Smoothie, Innocent Pomegranate, Blueberry and Acai Smoothie (Innocent Drinks, Dublin, Ireland). Samples were aliquoted into 25 mL centrifuge tubes and stored at $-20\text{ }^{\circ}\text{C}$ until analysis. For fortified samples, BSG phenolic extracts, P2 and B2, were added at 2.5 and 10 % (v/v) to a final volume of 6 mL prior to freezing. Previous research has been carried out using BSG phenolic extracts at a non-toxic concentration of 2.5 % (v/v) in cellular bioactivity assays (McCarthy *et al.*, 2012). Fortification of foods with whole BSG (ranging from 0 – 30 %) has also been carried out (Stojceska & Ainsworth, 2008; Özvural *et al.*, 2009; Öztürk *et al.*, 2002), however due to varying effects on consumer acceptability, a maximum level of addition of 10 % BSG phenolic extracts was chosen. All samples were analysed before and after *in vitro* digestion.

3.3.8. *In vitro* digestion of fruit beverages

Fruit beverage samples were subjected to *in vitro* digestion, using a procedure described elsewhere (O'Connell *et al.*, 2007). Briefly, sample (1 mL) was diluted to a volume of 10 mL with Hank's balanced salt solution (HBSS) and digested with pepsin (0.04 g/mL) at pH 2 and 37 °C for 1 hr in a shaking waterbath. Bile salts, consisting of glycodeoxycholate (0.8 mmol/L), taurodeoxycholate (0.45 mmol/L), taurocholate (0.75 mmol/L), and pancreatin (0.08 g/ml) were added, followed by a digestion period of 2 hr at 37 °C and pH 7.4 in a shaking waterbath. Samples were then ultracentrifuged at 53000 rpm for 95 min and the aqueous supernatant (digestate) was filtered through a 0.22 µm filter, overlaid with a layer of nitrogen and stored at -80 °C until further analysis. The TPC, DPPH radical scavenging and FRAP were measured on all beverages prior to and following *in vitro* digestion.

3.3.9. Analysis of the antioxidant potential of fruit beverages

The TPC and DPPH assays were conducted as detailed above. The ferric reducing antioxidant power (FRAP) assay (Benzie & Strain, 1996), which is based on the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) was used to determine the reducing power of the samples. The reduction of Fe^{3+} to Fe^{2+} is measured spectrophotometrically at 593 nm. To allow for coloured samples, colour blanks were used consisting of 100 µL sample and 2.9 mL distilled H_2O . For calibration, 0 - 100 µM ferrous sulphate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) was used and results were expressed as µM FRAP.

3.3.10. Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 4.00 (GraphPad Software, San Diego, California, USA). Data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett's test, or Tukey's test where stated, to determine significant ($P <$

0.05) differences between samples. The paired t-test was used for correlation statistics, with $P < 0.05$ indicating significant correlations.

3.4. Results

3.4.1. Hydroxycinnamic acid content of barley, BSG and phenolic extracts

The total hydroxycinnamic acid content was similar for both pale and black barley samples; having 1259.04 and 1164.34 $\mu\text{g/g}_{\text{dw}}$, respectively (Table 1). The total hydroxycinnamic acid content of barley was higher than its corresponding BSG for pale and black barley samples. Of the identified compounds, ferulic acid was consistently present in highest amounts, followed by *p*-coumaric acid derivatives and ferulic acid derivatives. Both barley samples contained similar concentrations of the three predominant hydroxycinnamic acids. Pale BSG had higher concentrations of individual and total hydroxycinnamic acids compared to black BSG.

The hydroxycinnamic acid contents of BSG phenolic extracts (P1 - P4; B1 - B4) were also quantified (Table 2). Caffeic acid derivatives were the only identifiable hydroxycinnamic acids present in extract P1 and B1. Extracts P2 - P4 and B2 - B4 all contain ferulic acid and its derivatives, and *p*-coumaric acid derivatives. The pale BSG extract P4 also contains sinapic acid (20.78 $\mu\text{g/mL}$). Caffeic acid was not detected in any of the BSG phenolic extracts, and caffeic acid derivatives, which were present in lowest concentrations in barley and BSG samples were not detected in extracts P2 - P4 or B2 - B4. Similar to their barley equivalents, the pale BSG phenolic extracts contained higher concentrations of individual and total hydroxycinnamic acids, compared to the black BSG phenolic extracts. The total hydroxycinnamic acid content of P2 (pale BSG) was more than 10 fold higher than that of B2 (black BSG). The hydroxycinnamic acid profile was altered in the phenolic extracts in comparison to the parent barley and BSG

Table 1: The hydroxycinnamic acid content of whole ground barley and whole brewers' spent grain (BSG) samples.

Hydroxycinnamic acid	Concentration ($\mu\text{g}/\text{g}_{\text{dw}}$)			
	Pale Barley	Pale BSG	Black Barley	Black BSG
Ferulic acid	352.13 \pm 51.57	142.29 \pm 24.17*	338.05 \pm 7.71	62.92 \pm 13.46*
Ferulic acid derivatives	100.40 \pm 30.33	66.37 \pm 1.75	92.79 \pm 4.23	27.56 \pm 2.79
Caffeic acid	8.42 \pm 1.02	2.78 \pm 0.15*	7.44 \pm 0.22	1.33 \pm 0.67*
Caffeic acid derivatives	7.91 \pm 0.88	3.64 \pm 1.28	4.33 \pm 1.88	3.68 \pm 1.48
<i>p</i> -Coumaric acid	67.45 \pm 0.97	42.66 \pm 0.13* [#]	66.66 \pm 0.79	18.83 \pm 0.34*
<i>p</i> -Coumaric acid derivatives	126.81 \pm 1.45	70.90 \pm 0.85* [#]	122.27 \pm 2.21	27.98 \pm 1.53*
Unknown	595.92 \pm 12.29	226.64 \pm 7.72* [#]	532.79 \pm 5.06	95.38 \pm 3.29*
Total	1259.04 \pm 69.26	555.29 \pm 29.99* [#]	1164.34 \pm 12.36	237.70 \pm 23.55*

Data represents the mean \pm SE of two independent experiments. Statistical analysis by ANOVA followed by Tukey's multiple comparison test. * Denotes statistically significant ($P < 0.01$) difference between value for whole BSG and corresponding barley sample. [#] Denotes statistically significant ($P < 0.05$) difference between value for pale BSG and corresponding value for black BSG.

Table 2: The hydroxycinnamic acid content of BSG phenolic extracts (P1 - P4, B1 - B4).

Hydroxycinnamic acid	Concentration ($\mu\text{g/mL}$)							
	P1	P2	P3	P4	B1	B2	B3	B4
FA	-	534.28 \pm 9.07*	1306.06 \pm 32.25*	563.50 \pm 20.89*	-	169.62 \pm 1.18	176.50 \pm 8.59	129.81 \pm 26.75
FA deriv.	-	779.90 \pm 13.69*	363.05 \pm 10.50*	289.25 \pm 13.23*	-	26.81 \pm 1.87	30.55 \pm 0.88	26.75 \pm 0.32
CA	-	-	-	-	-	-	-	-
CA deriv.	19.04 \pm 0.07*	-	-	-	4.27 \pm 3.03	-	-	-
<i>p</i> -CA	-	-	-	-	-	-	-	-
<i>p</i> -CA deriv.	-	2977.67 \pm 4.77*	845.23 \pm 1.68*	96.40 \pm 0.26	-	212.63 \pm 15.95	256.01 \pm 2.60	53.16 \pm 0.14
SA	-	-	-	20.78 \pm 0.04	-	-	-	-
Unknown	11.58 \pm 0.32*	723.63 \pm 19.01*	270.15 \pm 18.54*	121.05 \pm 11.52	22.89 \pm 1.20	71.48 \pm 5.07	42.99 \pm 0.61	103.53 \pm 6.78
Total	30.62 \pm 0.39	5015.49 \pm 19.16*	2784.50 \pm 4.90*	1090.98 \pm 4.17*	27.16 \pm 1.83	480.54 \pm 21.72	506.04 \pm 9.70	313.25 \pm 12.63

Data represents the mean \pm SE of two independent experiments. Statistical analysis by ANOVA followed by Tukey's multiple comparison test. * Denotes statistically ($P < 0.01$) significant difference between value and corresponding value for black BSG phenolic extracts. CA, caffeic acid; deriv, derivatives; FA, ferulic acid; *p*-CA, *p*-coumaric acid; SA, sinapic acid.

particularly in samples P2, B2 and B3 and the predominant hydroxycinnamic acids were the *p*-coumaric acid derivatives and not ferulic acid.

3.4.2. TPC and DPPH radical scavenging of barley, BSG and phenolic extracts

The TPC of the barley and BSG samples ranged from 1.26 - 4.53 mg GAE/g (Table 3). In general, the TPC trend as determined by Folin-Ciocalteu was similar to the trend of total hydroxycinnamic acid content of the barley and BSG samples as determined by HPLC with the exception of the black BSG which had the lowest total hydroxycinnamic acid content and the highest TPC. The DPPH scavenging activity ranged from 10.89 % for black barley to 17.18 % for pale barley (Table 3). The BSG phenolic rich extracts had TPC in the order P2 / B2 > P3 / B3 > P4 / B4 > P1 / B1 (Table 3). Correlation statistics suggest a significant ($P < 0.05$) correlation between DPPH inhibition and both caffeic acid ($R^2 = 0.8309$) and total hydroxycinnamic acid ($R^2 = 0.3942$) concentrations.

3.4.3. Antioxidant potential of fortified fruit beverages before and after digestion

The TPC of the juices and smoothies ranged from 0.37 to 2.15 mg GAE/mL (Table 4). The addition of BSG phenolic extracts P2 and B2 to the juices and smoothies at concentrations of 2.5 % and 10 % did not significantly enhance their TPC (Table 4). The addition of 2.5 % and 10 % P2 equates to the addition of 125.39 μ g and 501.55 μ g hydroxycinnamic acids per ml of juice, respectively. The addition of 2.5 % and 10 % B2 equates to the addition of 12.01 μ g and 48.05 μ g hydroxycinnamic acids per mL of juice, respectively. The TPC of the pomegranate smoothie was significantly decreased ($P < 0.05$) due to dilution with the phenolic extracts which had a lower TPC than the smoothie itself. The DPPH scavenging activity of the smoothies (10.95 and 11.11 %) was also higher than that of the juices (7.18 and 8.08 %). Addition of the phenolic extracts did not significantly alter the scavenging activity of the juices and smoothies (Table 4).

Table 3: Total phenolic content (TPC) and DPPH radical scavenging activity of whole ground barley, whole brewers' spent grain (BSG) and phenolic enriched extracts of BSG.

Sample	TPC (mg GAE/g _{dw})	DPPH scavenging (%)
Pale barley	2.08 ± 0.06	17.18 ± 0.69
Pale BSG	1.26 ± 0.10* [#]	14.19 ± 0.91
Black barley	1.88 ± 0.14	10.89 ± 2.64
Black BSG	4.53 ± 0.16*	17.01 ± 5.11
	(mg GAE/mL)	
P1	0.01 ± 0.01	14.88 ± 0.06
P2	0.34 ± 0.01	7.35 ± 1.77
P3	0.17 ± 0.01	6.78 ± 1.98
P4	0.05 ± 0.00	5.33 ± 1.42
B1	0.06 ± 0.00	5.81 ± 0.40
B2	0.50 ± 0.02 [†]	12.62 ± 1.83
B3	0.19 ± 0.01	10.17 ± 2.21
B4	0.14 ± 0.01 [†]	9.10 ± 2.03

Data represents mean ± SE of four independent experiments. Statistical analysis by ANOVA followed by Tukey's multiple comparison test. * Denotes statistically significant (P < 0.01) difference between TPC of whole BSG and corresponding barley sample. [#] Denotes statistically significant (P < 0.001) difference between TPC of pale BSG and black BSG. [†] Denotes statistically significant (P < 0.001) difference between TPC of black BSG extract and corresponding pale BSG extracts.

Table 4: Antioxidant activity of fruit beverages supplemented with BSG phenolic extracts.

Sample		TPC (mg GAE/mL)	DPPH scavenging (%)	FRAP (μ M)
Grape Juice	Control	0.66 \pm 0.07	8.08 \pm 1.19	144.87 \pm 31.45
	2.5 % (v/v) P2	0.54 \pm 0.07	7.77 \pm 1.85	130.82 \pm 23.13
	10 % (v/v) P2	0.65 \pm 0.09	7.92 \pm 1.22	89.05 \pm 22.41
	2.5 % (v/v) B2	0.47 \pm 0.06	6.10 \pm 1.21	112.12 \pm 19.34
	10 % (v/v) B2	0.91 \pm 0.18	6.15 \pm 0.12	149.73 \pm 16.87
Cranberry Juice	Control	0.37 \pm 0.03	7.18 \pm 0.34	118.07 \pm 41.72
	2.5 % (v/v) P2	0.54 \pm 0.12	9.65 \pm 2.03	217.77 \pm 34.23*
	10 % (v/v) P2	0.53 \pm 0.05	8.67 \pm 1.47	260.57 \pm 37.72*
	2.5 % (v/v) B2	0.41 \pm 0.03	7.99 \pm 1.48	266.07 \pm 34.23*
	10 % (v/v) B2	0.48 \pm 0.01	7.99 \pm 1.13	256.21 \pm 29.91*
Strawberry Smoothie	Control	1.24 \pm 0.01	10.95 \pm 1.20	399.72 \pm 35.62
	2.5 % (v/v) P2	1.27 \pm 0.03	10.28 \pm 1.12	407.48 \pm 37.37
	10 % (v/v) P2	1.43 \pm 0.19	8.33 \pm 0.93	391.23 \pm 42.73
	2.5 % (v/v) B2	1.33 \pm 0.15	9.22 \pm 1.69	430.66 \pm 48.73
	10 % (v/v) B2	1.29 \pm 0.07	9.22 \pm 0.18	439.30 \pm 69.00
Pomegranate Smoothie	Control	2.15 \pm 0.15	11.11 \pm 0.53	545.15 \pm 82.96
	2.5 % (v/v) P2	1.63 \pm 0.06*	8.71 \pm 2.11	516.64 \pm 82.44
	10 % (v/v) P2	1.66 \pm 0.15*	12.15 \pm 1.09	512.80 \pm 86.18
	2.5 % (v/v) B2	1.54 \pm 0.05*	9.83 \pm 0.06	513.27 \pm 83.61
	10 % (v/v) B2	1.49 \pm 0.12*	10.87 \pm 1.57	506.28 \pm 64.08

Data represents the mean \pm SE of at least three independent experiments. Statistical analysis by ANOVA followed by Tukey's multiple comparison test. * Denotes statistically significant ($P < 0.05$) difference between supplemented and unsupplemented beverage. Values in brackets represent the antioxidant activity, expressed as % of fruit beverage controls.

The FRAP activity of the unsupplemented juices and smoothies ranged from 118.07 to 545.15 μM FRAP. FRAP activity was significantly increased ($P < 0.05$) in the cranberry juice with added BSG phenolic extracts compared to the unsupplemented cranberry juice (Table 4).

Following *in vitro* digestion, no significant difference in TPC, DPPH radical scavenging or FRAP between the juices or smoothies with or without the addition of phenolic extracts was found (Table 5). The DPPH radical scavenging activity was similar for both the juices and the smoothies after digestion, however, FRAP values remained higher in the smoothies than in the juices following digestion. *In vitro* digestion resulted in significant ($P < 0.05$) decreases in bioactivity of the juices and smoothies samples.

3.5. Discussion

The predominant hydroxycinnamic acid found in each of the barley samples was ferulic acid and its concentration ranged from 338.05 - 352.13 $\mu\text{g/g}_{\text{dw}}$ (Table 1). This concentration of ferulic acid was higher than that previously reported for barley (159.70 - 231.30 $\mu\text{g/g}_{\text{dw}}$) (Dvorakova *et al.*, 2008). Similar to a previous study, the concentration of hydroxycinnamic acids present in barley was in the order of ferulic acid > *p*-coumaric acid > caffeic acid (Dvorakova *et al.*, 2008). In addition, greater quantities of derivatives of ferulic and *p*-coumaric acid were found in comparison to caffeic acid derivatives. Phenolic acid derivatives present in both barley and BSG seem to be glycosides with pentoses and hexoses. While the profile of hydroxycinnamic acids present in barley has been found to be similar in a number of studies (Dvorakova *et al.*, 2008; Holtekjølen *et al.*, 2006), there was considerable variation in the total quantities measured which could result from the difference in barley cultivars (Holtekjølen *et al.*, 2006; Zhao *et al.*, 2008) and more importantly the presence or absence of hull (Holtekjølen *et al.*, 2006).

Table 5: Antioxidant activity of fruit beverages supplemented with BSG phenolic extracts, following *in vitro* digestion.

Sample		TPC (mg GAE/mL)	DPPH scavenging (%)	FRAP (μ M)
Grape	Control	0.21 \pm 0.02*	10.06 \pm 2.83*	7.35 \pm 1.95*
Juice	2.5 % (v/v) P2	0.19 \pm 0.02	14.62 \pm 3.25*	6.60 \pm 2.23*
	10 % (v/v) P2	0.20 \pm 0.01	12.87 \pm 2.39*	6.23 \pm 1.27*
	2.5 % (v/v) B2	0.18 \pm 0.01	12.79 \pm 2.80*	5.88 \pm 1.49*
	10 % (v/v) B2	0.18 \pm 0.01*	12.55 \pm 3.01*	5.24 \pm 1.40*
	Control	0.17 \pm 0.03	9.50 \pm 1.96	8.18 \pm 2.09*
Cranberry	2.5 % (v/v) P2	0.19 \pm 0.00*	9.13 \pm 6.94	7.49 \pm 2.87*
	10 % (v/v) P2	0.20 \pm 0.01*	14.46 \pm 2.39*	7.88 \pm 2.06*
	2.5 % (v/v) B2	0.18 \pm 0.02	14.28 \pm 3.33	4.41 \pm 2.00*
	10 % (v/v) B2	0.12 \pm 0.05	14.48 \pm 3.00	3.26 \pm 0.92*
	Control	0.20 \pm 0.02*	11.46 \pm 1.87*	13.85 \pm 2.32*
Strawberry	2.5 % (v/v) P2	0.20 \pm 0.01*	10.62 \pm 3.63*	10.22 \pm 1.06*
	10 % (v/v) P2	0.22 \pm 0.01*	8.06 \pm 1.70*	12.81 \pm 2.44*
	2.5 % (v/v) B2	0.23 \pm 0.02*	11.02 \pm 3.79*	10.59 \pm 0.47*
	10 % (v/v) B2	0.22 \pm 0.03*	6.91 \pm 0.82*	9.77 \pm 0.35*
	Control	0.20 \pm 0.03*	11.84 \pm 2.74*	19.35 \pm 2.20*
Pomegranate	2.5 % (v/v) P2	0.20 \pm 0.01*	5.50 \pm 4.01*	13.97 \pm 3.65*
	10 % (v/v) P2	0.19 \pm 0.00*	10.28 \pm 2.27*	12.43 \pm 0.84*
	2.5 % (v/v) B2	0.20 \pm 0.00*	9.13 \pm 2.67*	17.09 \pm 2.55*
	10 % (v/v) B2	0.20 \pm 0.00*	10.43 \pm 1.60*	15.20 \pm 1.76*
	Control	0.20 \pm 0.03*	11.84 \pm 2.74*	19.35 \pm 2.20*

Data represents the mean \pm SE of at least three independent experiments. * Denotes statistically significant ($P < 0.05$) difference between undigested (Table 4) and digested values.

Environmental factors, including soil type, sun exposure and climatic conditions, also affect polyphenol concentration (Manach *et al.*, 2004). The order of hydroxycinnamic acids detected in BSG was in similar order to those present in barley, as outlined above. These support previous findings regarding phenolic acids in BSG, where ferulic acid was found to be in highest abundance followed by *p*-coumaric, sinapic and caffeic acids (Dvorakova *et al.*, 2008).

Extracts P1 and B1, which are aqueous extracts of BSG, were the only extracts found to contain caffeic acid derivatives (Table 2). This suggests that the polar caffeic acid derivatives in BSG are in the free form, since aqueous extraction releases the free, water-soluble phenolics. Caffeic acid derivatives present may be isomers esterified with syringic acid, a hydroxybenzoic acid, present in BSG (Mussatto *et al.*, 2007). The presence of caffeic acid derivatives at higher concentrations in P1 compared to B1 may be responsible for the high antioxidant activity shown by extract P1, as caffeic acid has previously been shown to have higher antioxidant activity than other hydroxycinnamic acids (Kikuzaki *et al.*, 2002). Extracts P2 - P4 and B2 - B4 contain the bound phenolic compounds, released with sodium hydroxide (McCarthy *et al.*, 2012). Extraction with 1N NaOH resulted in extracts with highest HA concentrations, for example, extracts P2/B2 and P3/B3. Ferulic acid, ferulic acid derivatives (assuming dehydrodiferulic acid isomers) and *p*-coumaric acid derivatives were the hydroxycinnamic acids present in these extracts, suggesting they are present in the bound form in BSG. It has been reported that extraction with aqueous HCl alone destroys hydroxycinnamic acids, while ethanol extraction can give low yield (Gao & Mazza, 1994).

It is evident that the brewing process causes a reduction in hydroxycinnamic acid concentration as barley had a higher content of hydroxycinnamic acids than BSG (Table 1). Furthermore, differences in hydroxycinnamic acid content between pale BSG and black BSG suggest that roasting of the grain, in the case of black BSG, reduces the

concentration of hydroxycinnamic acids present. Black BSG had higher TPC and DPPH activity than barley and pale BSG samples (Table 3). The Folin-Ciocalteu method is not specific to phenolic compounds, but measures any compound which is capable of reducing FCR (Huang *et al.*, 2005). Increased antioxidant activity may be attributable to non-enzymatic browning Maillard reaction products (MRPs), which can act as antioxidants (Samaras *et al.*, 2005). Similarly, the roasting of coffee results in the transformation of phenolic compounds to brown high molecular weight MRPs including melanoidins (del Castillo *et al.*, 2002). Results of the present study support previous findings that although thermal treatments can reduce the concentration of natural antioxidants in a product, the overall antioxidant activity of the product is maintained or enhanced through production of MRPs (Nicoli *et al.*, 1997).

Caffeic acid and total hydroxycinnamic acid concentrations of the barley, BSG and extracts were correlated with DPPH radical scavenging ability ($R^2 = 0.86309$ and $R^2 = 0.3942$, respectively, $P < 0.05$). Evidence in the literature is contradictory regarding the phenolic profile of substances and their antioxidant activity. Some studies report close correlations between TPC and DPPH scavenging (Lee *et al.*, 2010; Zhao *et al.*, 2008), while others have reported the absence of a correlation between antioxidant capacity and hydroxycinnamic acid content (Chinnici *et al.*, 2004) or TPC (Heimler *et al.*, 2006). It has been suggested that interference by other substances can affect the correlation between radical scavenging activity and phenolic content (Stratil *et al.*, 2006), since the TPC assay measures the presence of any reducing agent including inorganic ions, vitamins and nitrogen containing compounds and not just phenolic compounds (Huang *et al.*, 2005).

Phenolic rich extracts P2 and B2 which had the highest hydroxycinnamic acid contents of the BSG extracts (Table 2) were selected for addition to fruit juices and smoothies in order to determine if the antioxidant potential of the juices could be enhanced before and after an *in vitro* digestion procedure.

White grape juice was found to have a higher antioxidant activity than cranberry juice (Table 4). The antioxidant activity of the smoothies was higher than that of the fruit juices. It has been suggested that combinations of different phenolic compounds, as is the case in fruit smoothies, can result in a synergistic antioxidant effect (Cirico & Omaye, 2006). Results from the present study showed that the addition of the BSG phenolic extracts (P2 and B2) did not significantly increase TPC or the DPPH radical scavenging activity of the juices and smoothies as the extracts had a similar TPC to the fruit juices and a lower TPC than the smoothies (Table 4). However, there was a significant increase in the FRAP activity of the cranberry juice with added phenolic extracts, possibly due to interactions between different phenolic compounds which can result in a synergistic antioxidant effect, as previously mentioned (Cirico & Omaye, 2006).

The hydroxycinnamic acid in highest concentration in BSG is ferulic acid (Dvorakova *et al.*, 2008). Ferulic acid was found in the phenolic extracts used for this study, P2 and B2, at levels of 534.28 ± 9.07 and 169.62 ± 1.18 $\mu\text{g/ml}$, respectively (Table 2). It has been shown that ferulic acid can interact synergistically with other antioxidants including ascorbic acid, α -tocopherol and β -carotene (Trombino *et al.*, 2004).

It has been reported that antioxidant activity of selected fruit juices (cranberry, red grape, pomegranate and long life orange juices) increases following digestion, possibly due to an increase in anthocyanins (Ryan & Prescott, 2010). However, it has also been shown that the *in vitro* digestion of fruit beverages can decrease the phenolic concentration, due to alkaline conditions and interactions between polyphenols and enzymes (Cilla *et al.*, 2009). Stalmach *et al.* (2012) showed that hydroxycinnamic acids are relatively stable during simulated gastric and duodenal digestion, in comparison with other polyphenolic compounds, with an average recovery of 82 % following *in vitro* digestion. Therefore, we postulated that addition of BSG extracts to fruit juices may result in an increased antioxidant activity following digestion as the stability of the added

BSG phenolics, which have a high content of hydroxycinnamic acids, may be greater than that of the polyphenols inherent in the fruit juices during the digestive process. However, under the conditions of the present experiment there were no significant differences in the antioxidant activity of fruit beverages with or without added BSG phenolics following an *in vitro* digestion procedure. It is noteworthy that the protective effects of phenolic compounds, including antioxidant, can occur within the gastrointestinal (GI) tract before absorption (Halliwell *et al.*, 2000). This may account for the ability of foods rich in phenolic compounds to protect against gastric and oral cancers, for example catechins from tea can arrest growth and induce apoptosis in oral cancer (Masuda *et al.*, 2001). The benefits of supplementation with BSG may be more apparent in a food system with a lower hydroxycinnamic acid or total polyphenol content.

3.6. Conclusion

This study has shown the main hydroxycinnamic acids in barley and BSG to be ferulic and *p*-coumaric acid and their derivatives. The bound hydroxycinnamic acids in BSG are ferulic acid and *p*-coumaric acid derivatives, which are released by alkaline extraction. Caffeic acid was the only free phenolic acid identified and was significantly correlated to DPPH scavenging activity, as was total hydroxycinnamic acid content. In addition, it was shown that the brewing process decreases the amount of hydroxycinnamic acids present. Roasting of the barley grain (to produce black BSG) resulted in a greater decrease in hydroxycinnamic acid content, compared to unroasted, while the greater antioxidant activity in black BSG may be attributable to MRPs. Furthermore, the present study highlights the potential of BSG phenolic extracts to be incorporated as antioxidants into functional foods, significantly increasing FRAP activity of cranberry juice. This study warrants further investigation of the fortification of foods with BSG phenolic extracts. It may be that more beneficial effects may be observed if these extracts are added to foods

with a lower antioxidant profile. The application of BSG phenolic extracts in food formulation presents a means of utilising this co-product, thus reducing brewery waste, while also providing a novel source of functional food ingredients for the food industry.

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Chapter 4

Phenolic enriched fractions from brewers' spent grain (BSG) possess cellular antioxidant and immunomodulatory effects in cell culture model systems.

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4.1. Abstract

Large quantities of brewers' spent grain (BSG), a co-product of the brewing industry, are produced annually. BSG contains hydroxycinnamic acids, and phenolic-rich extracts from BSG have previously demonstrated the ability to protect against oxidant-induced DNA damage. The present study investigated the anti-inflammatory potential of eight phenolic extracts from BSG, four pale (P1 - P4) and four black (B1 - B4) extracts.

BSG extracts were more cytotoxic in Jurkat T than U937 cells, with lower IC₅₀ values in Jurkat T cells, measured using the (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Pale BSG extracts P2 and P3 showed the greatest anti-inflammatory potential; significantly ($P < 0.05$) reducing interleukin-2 (IL-2), interleukin-4 (IL-4, P2 only), interleukin-10 (IL-10) and interferon- γ (IFN- γ) production. In addition, extracts P1 - P3 and B2 - B4 showed significant ($P < 0.05$) antioxidant effects, determined by the cellular antioxidant activity assays: superoxide dismutase (SOD), catalase (CAT) and glutathione content (GSH).

Phenolic extracts from BSG, particularly the pale BSG extracts, have the ability to reduce a stimulated cytokine production and may also protect against cellular oxidative stress. Results of the present study highlight the potential of BSG phenolic extracts to act as functional food ingredients, providing an alternative use and improving the value of this brewing industry co-product.

4.2. Introduction

Beer is the fifth most consumed beverage in the world after tea, carbonates, milk and coffee (Fillaudeau *et al.*, 2006), with an average consumption in Europe of 72.8 litres per capita in 2011 (The Brewers of Europe, online article). Brewers' spent grain (BSG) is a solid co-product of the brewing industry, corresponding to around 85 % of total brewing by-products (Reinold, 1997). Currently, the utilisation of BSG is mainly restricted to

animal feed, particularly for dairy cattle. However, due to the large quantities produced and increasing transport costs, alternative uses are sought. BSG has a number of potential applications including energy production (direct combustion or fertilisation to produce a biogas), biotechnological processes (substrates for microbial cultivation and enzyme production) and human nutrition (Mussatto *et al.*, 2006). Due to its low cost and favourable nutritional composition (20 % protein, 70 % fibre; Mussatto *et al.*, 2006), BSG has been investigated as a potential ingredient for human nutrition. Both pale and black (where barley is roasted to 200 °C prior to brewing) BSG are sources of hydroxycinnamic acids, particularly ferulic and *p*-coumaric acids and their derivatives, with total hydroxycinnamic acid contents of 555.29 and 237.70 µg/g_{dw}, respectively (McCarthy *et al.*, 2013).

Immunomodulatory agents have the ability to influence the immune response, either positively or negatively (Pragasam *et al.*, 2013). Inflammation has been linked to atherosclerosis (Berliner *et al.*, 1995), Alzheimer's (Salloway *et al.*, 2008) and Parkinson's disease (Hald & Lotharius, 2005), and immunosuppressants are now widely used in the treatment of inflammation and autoimmune diseases (Pragasam *et al.*, 2013). For this reason, plant-derived bioactive compounds that possess anti-inflammatory properties are in demand.

In addition to their anti-inflammatory potential, the hydroxycinnamic acids present in BSG also possess antioxidant capabilities (Nardini *et al.*, 1995; Chen & Ho, 1997; McCarthy *et al.*, 2012). Cellular antioxidant enzymes, such as catalase (CAT) and superoxide dismutase (SOD), neutralise reactive oxygen species (ROS). CAT is found in humans at highest levels in the liver, kidney and erythrocytes and detoxifies hydrogen peroxide (H₂O₂) to oxygen and water. SODs are a group of metalloenzymes that catalyse the dismutation of the superoxide anion (O₂^{•-}) at an extremely fast rate, keeping concentrations in cells and tissues very low. SOD forms a crucial part of the cellular

antioxidant defence mechanism. Under conditions of oxidative stress the activity of antioxidant enzymes can be altered, owing to adaptation of the cellular antioxidant system to ROS production (Mansour *et al.*, 2011).

The ability of phenolic extracts from BSG to protect against oxidant-induced DNA damage *in vitro* has been previously demonstrated (McCarthy *et al.*, 2012). The aim of the present study was to determine the immunomodulatory potential of eight phenolic extracts from BSG by measuring the production of four cytokines: interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-10 (IL-10) and interferon- γ (IFN- γ), using an enzyme-linked immunosorbent assay (ELISA) in the Jurkat T cell line (human leukaemic T cell line). In addition, the ability of BSG phenolic extracts to protect against oxidation induced by H₂O₂ in a human monocytic blood cell line (U937 cells) was measured using the CAT, GSH and SOD assays.

4.3. Materials and Methods

4.3.1. Materials

All chemicals and reagents were purchased from Sigma-Aldrich Ireland Limited, Co. Wicklow, Ireland, unless otherwise stated. U937 and Jurkat T cells were obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, Wilts, UK).

4.3.2. Generation of phenolic extracts from BSG

A total of eight phenolic extracts were prepared from pale (P1 - P4) and black (B1 - B4) BSG, as described previously (McCarthy *et al.*, 2012). Briefly, P1 and B1 were the supernatants produced by centrifugation, following shearing in H₂O. The precipitate was subjected to 110 mM NaOH extraction and following centrifugation, the supernatants were designated P4 and B4. Extracts P2 and B2 were the supernatant fractions obtained by 110 mM and 1N NaOH extraction followed by centrifugation. Sediment remaining

from this step was washed with H₂O to yield extracts P3 and B3. All samples were adjusted to pH 7.0 prior to analysis, using either 2 N NaOH or 2 N HCl, as appropriate.

4.3.3. Cell culture

A leukaemic monocytic lymphoma cell line, U937 cells, and a human leukaemic T cell line, Jurkat T cells, were maintained at 37 °C in a 5 % CO₂ atmosphere, in antibiotic-free medium (RPMI-1640) supplemented with 10 % foetal bovine serum (FBS). For experiments, reduced serum media was used (2.5 % FBS). Cells were adjusted to a density of either 1 x 10⁵ cells/mL or 2 x 10⁵ cells/mL, as appropriate, for each experiment.

4.3.4. Cell viability assay

Viability of U937 and Jurkat T cell lines was measured by the (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to determine subtoxic concentrations of the BSG phenolic extracts for subsequent experiments. U937 or Jurkat T cells (2 x 10⁵ cells/mL) were exposed to BSG phenolic extracts at concentrations of 0 - 20 % and 0 - 10 % (v/v), respectively, for 24 hr at 37 °C in a 96 well plate. Following incubation, an MTT assay kit (MTT I proliferation kit, Roche Diagnostics, UK) was used to carry out the assay. MTT reagent 1 (10 µL) was added and cells were incubated for a further 4 hr at 37 °C. MTT reagent 2 (100 µL) was then added and cells were incubated for 24 hr at 37 °C. The absorbance was measured on a microplate reader (ThermoScientific Varioskan Flash) at 570 nm. A media blank (no cells) was used to correct for the background colour of the BSG samples. The half maximal inhibitory concentration (IC₅₀) values were determined using GraphPad Prism 4 and expressed as % (v/v). Using data from previous work (McCarthy *et al.*, 2012), coupled with results of the MTT assay and IC₅₀ values, a

concentration of 2.5 % (v/v) BSG phenolic extracts was selected for further analysis in the U937 cells. A concentration of 5 % (v/v) BSG phenolic extracts was selected for addition to the Jurkat T cells stimulated with concanavalin A (which stimulates cell proliferation and cytokine production). Cell viability was greater than 90 % for all cytokine assay conditions.

4.3.5. Enzyme-linked immunosorbent assay (ELISA)

Jurkat T cells (2×10^5 cells/mL) were incubated with BSG phenolic extracts (5 % v/v) in a 96 well plate for 24 hr at 37 °C, in the presence of Concanavalin A (conA, 50 µg/mL). ELISA kits (eBioscience Human Th1/Th2 ELISA Ready-SET-Go kit, Insight Biotechnology, UK) were used to determine the production of IL-2, IL-4, IL-10 and IFN- γ . Sample absorbance was measured at 450 nm on a microplate reader (ThermoScientific Varioskan Flash) and data were expressed as a percentage of the ConA stimulated Jurkat T cell control.

4.3.6. Antioxidant assays- superoxide dismutase, glutathione, catalase

U937 cells (1×10^5 cells/mL, 5 mL) were incubated with BSG phenolic extracts (2.5 % v/v) for 24 hr at 37 °C. Following incubation, cells were exposed to either 100 µM H₂O₂ (superoxide dismutase and glutathione assays) or 200 µM H₂O₂ (catalase assay) for 60 min. Cells were harvested, sonicated and centrifuged (800 rpm, 10 min) and the supernatant was collected for the determination of antioxidant enzyme activity.

SOD activity was measured using a Calbiochem SOD Assay Kit II (Merck Chemicals Ltd.) according to the protocol provided with the kit and sample absorbance was determined at 450 nm using a microplate reader (ThermoScientific Varioskan Flash). The GSH content of the cells was measured according to a method described previously (Hissin & Hilf, 1976). Briefly, 100 µL sample was mixed with sodium phosphate-

ethylenediamine tetraacetic acid buffer (1.8 mL) and σ -phthaldialdehyde (0.1 mg). The fluorescence intensity of the samples was determined (ThermoScientific Varioskan Flash microplate reader) at wavelengths of 350 nm (absorption) and 420 nm (emission). The GSH content of the samples was determined from a standard curve (0 - 25 μ M GSH). CAT activity was measured using a Calbiochem catalase assay kit (Merck Chemicals Ltd.). The absorbance was measured at 540 nm using a microplate reader (ThermoScientific Varioskan Flash) and a standard curve consisting of formaldehyde (0 - 75 μ M) was used for determining CAT activity. SOD, GSH and CAT activity were determined relative to the protein content as SOD units/mg protein in cell homogenate (U/mg protein), μ M GSH/mg protein and nmol CAT/min/ml, 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.

4.3.7. Statistical analysis

All data represent the mean \pm standard error (SE) of at least three independent experiments. Statistical analysis was carried out using GraphPad Prism version 4.00 for Windows (GraphPad software, San Diego, California, USA). Data was analysed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Statistical significance was determined at $P < 0.01$ or $P < 0.05$.

4.4. Results

4.4.1. Cell viability

The IC_{50} value indicates the concentration of a compound needed to reduce cell viability to 50 % of control, untreated cells. In general, BSG phenolic extracts were more cytotoxic in the Jurkat T cell line (Table 2) than in the U937 cell line (Table 1).

Therefore, IC₅₀ values were lower in the Jurkat T cells, where determinable; for example IC₅₀ values of 8.92 % (U937) versus 3.45 % (Jurkat T) and 5.23 % (U937) versus 2.48 % (Jurkat T) for P2 and B3, respectively (Table 3). Extract P4 was the least cytotoxic with 93 % U937 cell viability at 4 % (v/v) and 91 % Jurkat T cell viability at 8 % (v/v) (Tables 1 and 2). A non-toxic concentration of 2.5 % (v/v) was selected for analysis of the antioxidant activity in U937 cells. The Jurkat T cells were stimulated with conA to produce pro-inflammatory cytokines and incubated with 5 % (v/v) BSG phenolic extracts. Cell viability in Jurkat T cell co-incubated with ConA and 5 % (v/v) BSG phenolic extracts were not lower than 90 % (data not shown).

4.4.2. Immunomodulatory effects of BSG phenolic extracts

The immunomodulatory potential of phenolic extracts from BSG was determined by measuring their effect on the production of cytokines IL-2, IL-4, IL-10, IFN- γ in conA-stimulated Jurkat T cells (Table 4). Extract P2 significantly ($P < 0.05$) reduced the production of all cytokines investigated in the present study by at least 55.33 %, relative to the control cells. IFN- γ production was reduced to the greatest extent, with extract P2 reducing production to 20.05 % that of control conA-stimulated cells. Pale BSG extract, P3, also showed anti-inflammatory effects, significantly ($P < 0.05$) reducing the production of IL-2, IL-10 and IFN- γ to 57.67, 27.57 and 46.62 %, respectively. Extract B3 was the only black BSG extract which demonstrated a significant immunomodulatory effect, significantly ($P < 0.05$) reducing IFN- γ production to 55.82 % of the conA-stimulated, control cells.

Table 1: The effect of brewers' spent grain (BSG) phenolic extracts (0 – 20 % v/v) on cell proliferation in the U937 cell line.

Concentration (% v/v)	Cell Proliferation (% of control)							
	P1	P2	P3	P4	B1	B2	B3	B4
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 3.81	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.5	96.10 ± 2.02	102.57 ± 4.51	87.09 ± 5.77	100.69 ± 2.92	83.30 ± 8.83	101.23 ± 3.10	82.33 ± 4.25	98.57 ± 7.84
1	96.18 ± 1.45	103.53 ± 7.43	64.26 ± 7.45*	102.20 ± 8.31	67.04 ± 11.88*	94.13 ± 9.28	79.52 ± 5.67	89.88 ± 3.67
2	87.65 ± 0.72	100.74 ± 6.34	61.04 ± 6.25*	98.61 ± 5.65	53.41 ± 8.10*	68.85 ± 2.87	69.91 ± 2.83*	98.11 ± 4.82
2.5	73.28 ± 8.81*	104.54 ± 4.88	50.19 ± 7.47*	104.04 ± 6.04	50.81 ± 8.33*	55.31 ± 10.29*	47.81 ± 6.75*	90.41 ± 3.29
4	82.66 ± 1.28*	90.97 ± 2.23	59.04 ± 6.05*	93.08 ± 1.75	48.81 ± 6.12*	54.35 ± 8.84*	49.26 ± 5.59*	94.51 ± 2.92
5	84.47 ± 2.35	89.33 ± 6.62	59.44 ± 6.94*	86.84 ± 1.81	47.97 ± 4.49*	57.09 ± 8.50*	47.33 ± 4.89*	97.08 ± 4.56
6	76.79 ± 2.63*	61.64 ± 6.10*	54.08 ± 6.70*	79.58 ± 3.93*	40.53 ± 4.38*	49.56 ± 7.66*	34.54 ± 2.38*	89.99 ± 4.83
8	65.56 ± 6.37*	30.56 ± 5.88*	54.06 ± 5.71*	73.16 ± 6.63*	39.49 ± 4.17*	23.00 ± 4.78*	20.15 ± 6.46*	87.64 ± 3.02
10	64.38 ± 4.86*	2.02 ± 2.03*	56.36 ± 7.75*	69.72 ± 6.94*	34.52 ± 3.23*	23.91 ± 3.61*	1.36 ± 0.96*	76.31 ± 4.74*
20	49.58 ± 4.35*	5.50 ± 6.73*	17.42 ± 11.24*	59.56 ± 6.33*	31.29 ± 4.23*	33.12 ± 15.04*	11.33 ± 11.21*	60.20 ± 3.98*

Values represent the mean ± SE of four independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * Denotes statistically significant difference between sample and untreated cells control, P < 0.05.

Table 2: The effect of brewers' spent grain (BSG) phenolic extracts (0 – 10 % v/v) on cell proliferation in the Jurkat T cell line.

Concentration (% v/v)	Cell Proliferation (% of control)							
	P1	P2	P3	P4	B1	B2	B3	B4
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.5	99.55 ± 5.13	77.68 ± 6.34*	60.12 ± 7.94*	97.80 ± 3.89	73.95 ± 10.61*	95.61 ± 6.20	70.31 ± 8.00*	103.06 ± 6.81
1	99.81 ± 3.01	77.55 ± 2.46*	55.97 ± 11.35*	91.82 ± 5.32	67.19 ± 8.99*	89.95 ± 3.30	57.06 ± 8.38*	98.10 ± 7.74
2	93.63 ± 9.64	57.04 ± 11.55*	51.20 ± 14.44*	86.25 ± 14.39	54.68 ± 6.11*	77.82 ± 11.97*	52.08 ± 9.71*	83.42 ± 10.08
2.5	103.65 ± 10.82	59.42 ± 14.06*	53.00 ± 17.03*	98.90 ± 11.29	59.14 ± 4.06*	79.84 ± 2.53*	48.72 ± 9.17*	87.04 ± 9.83
4	99.10 ± 11.53	42.14 ± 14.44*	53.82 ± 15.46*	98.11 ± 15.58	59.68 ± 6.36*	68.54 ± 4.00*	20.21 ± 10.35*	86.16 ± 13.70*
5	93.94 ± 6.13	17.56 ± 11.85*	57.34 ± 19.89*	98.04 ± 16.00	59.56 ± 2.36*	73.51 ± 13.32*	9.80 ± 8.99*	84.96 ± 5.52
6	92.33 ± 4.35	13.34 ± 10.01*	51.99 ± 18.72*	100.02 ± 14.65	57.69 ± 2.79*	64.21 ± 8.55*	5.43 ± 1.04*	78.51 ± 8.04*
8	81.83 ± 8.78*	6.79 ± 2.14*	47.93 ± 11.86*	91.43 ± 17.47	53.56 ± 7.75*	48.69 ± 15.92*	4.81 ± 3.52*	79.14 ± 10.12*
10	74.85 ± 6.17*	5.48 ± 0.05*	27.52 ± 6.01*	83.98 ± 12.08	40.55 ± 9.28*	23.78 ± 16.48*	2.68 ± 7.93*	71.50 ± 12.63*

Values represent the mean ± SE of five independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * Denotes statistically significant difference between sample and untreated cells control, $P < 0.05$.

Table 3: The half maximal inhibitory concentration (IC_{50}) values of phenolic extracts from brewers' spent grain (BSG), in both the U937 and Jurkat T cell lines.

Sample	IC_{50} (expressed as % (v/v))	
	U937 cell line	Jurkat T cell line
P1	17.45	n/d
P2	8.92	3.45
P3	8.82	5.40
P4	20.89	n/d
B1	6.97	7.41
B2	8.09	7.22
B3	5.23	2.48
B4	26.09	n/d

n/d : IC_{50} value was not determinable from the range of concentrations tested.

Table 4: The effect of phenolic extracts from brewer's spent grain (5 % (v/v)) on cytokine production in concanavalin-A stimulated Jurkat T cells.

BSG sample	Cytokine production (% of control)			
	IL-2	IL-4	IL-10	IFN- γ
control	100.00 \pm 0.00	100.00 \pm 0.00	100.00 \pm 0.00	100.00 \pm 0.00
P1	89.34 \pm 3.31	85.90 \pm 4.99	72.96 \pm 9.13	82.80 \pm 10.06
P2	44.67 \pm 16.87 [#]	36.70 \pm 11.26*	39.15 \pm 16.36*	20.05 \pm 2.79 [#]
P3	57.67 \pm 6.25*	66.15 \pm 8.39	27.57 \pm 5.01 [#]	46.62 \pm 10.07 [#]
P4	90.87 \pm 8.71	97.46 \pm 8.22	56.60 \pm 13.09	60.79 \pm 7.55
B1	95.55 \pm 2.79	86.18 \pm 5.35	73.42 \pm 18.79	64.49 \pm 9.64
B2	92.82 \pm 9.64	97.84 \pm 3.58	127.20 \pm 11.35	76.86 \pm 11.36
B3	78.10 \pm 5.91	82.81 \pm 4.23	50.70 \pm 15.39	55.82 \pm 6.89*
B4	108.15 \pm 15.16	101.21 \pm 1.66	81.99 \pm 6.77	73.45 \pm 10.62

Values are mean \pm SE of four independent experiments, expressed as a percentage relative to cells treated with conA alone. Statistical analysis by ANOVA followed by Dunnett's test. * P < 0.01 [#] P < 0.05, statistically significant difference in cytokine production between control and cells treated with BSG phenolic extract.

4.4.3. Antioxidant potential of BSG phenolic extracts

SOD activity was significantly reduced ($P < 0.01$) to 55.36 % in U937 cells following the addition of 100 μM H_2O_2 for 60 min (Figure 1). Extracts P1, B2 and B4 significantly ($P < 0.05$) protected against the reduction in SOD activity which was at levels similar to control cells in the presence of P1 (98.92 %). An increase in activity above that of control cells was detected in cells treated with B2 and B4 (171.73 and 169.85 %, respectively). Similarly, exposure of U937 cells to 100 μM H_2O_2 for 60 min significantly ($P < 0.05$) reduced GSH content to 51.51 % of the control cells (Figure 2). Extracts from pale BSG demonstrated the greatest antioxidant effect and samples P1, P2 and P3 significantly ($P < 0.05$) protected against H_2O_2 -induced glutathione depletion. Extract B4 also significantly ($P < 0.05$) protected against glutathione depletion in U937 cells exposed to H_2O_2 . Pale BSG extracts (P1 - P3) and black BSG extracts (B2 and B3) also significantly ($P < 0.05$) protected against a H_2O_2 -induced decrease in CAT activity (Figure 3).

4.5. Discussion

Both pale and black BSG contain the hydroxycinnamic acids, ferulic, *p*-coumaric and caffeic acids, with total hydroxycinnamic acid contents of 555.29 and 237.70 $\mu\text{g}/\text{g}_{\text{dw}}$, respectively (McCarthy *et al.*, 2013) and phenolic extracts from BSG have previously demonstrated the ability to protect against oxidant-induced DNA damage (McCarthy *et al.*, 2012). This study aimed to determine if BSG phenolic extracts possess anti-inflammatory and antioxidant effects in Jurkat T and U937 cells, respectively. The MTT assay was used to determine the cytotoxicity of the BSG phenolic extracts (P1 - P4 and B1 - B4) in both cell lines. It was found that the samples were more cytotoxic in the

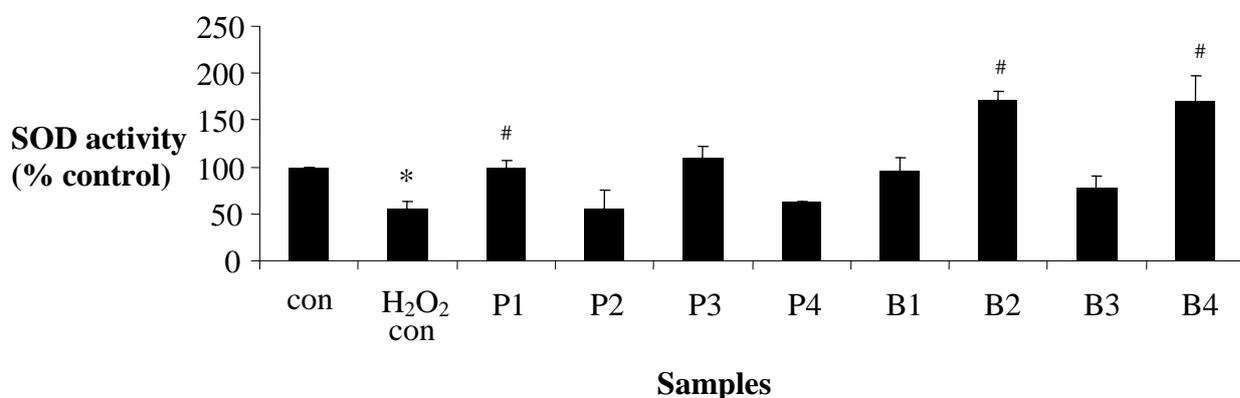


Figure 1: The antioxidant potential of pale (P) and black (B) brewers' spent grain (BSG) phenolic extracts, measured by their ability to protect against oxidative stress induced by hydrogen peroxide (H₂O₂) in the U937 cell line, using the superoxide dismutase (SOD) assay. Values represent the mean \pm SE of three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * Denotes statistically significant difference in SOD activity between control and H₂O₂ control, $P < 0.01$. # Denotes statistically significant difference in SOD activity between sample and H₂O₂ control, $P < 0.05$.

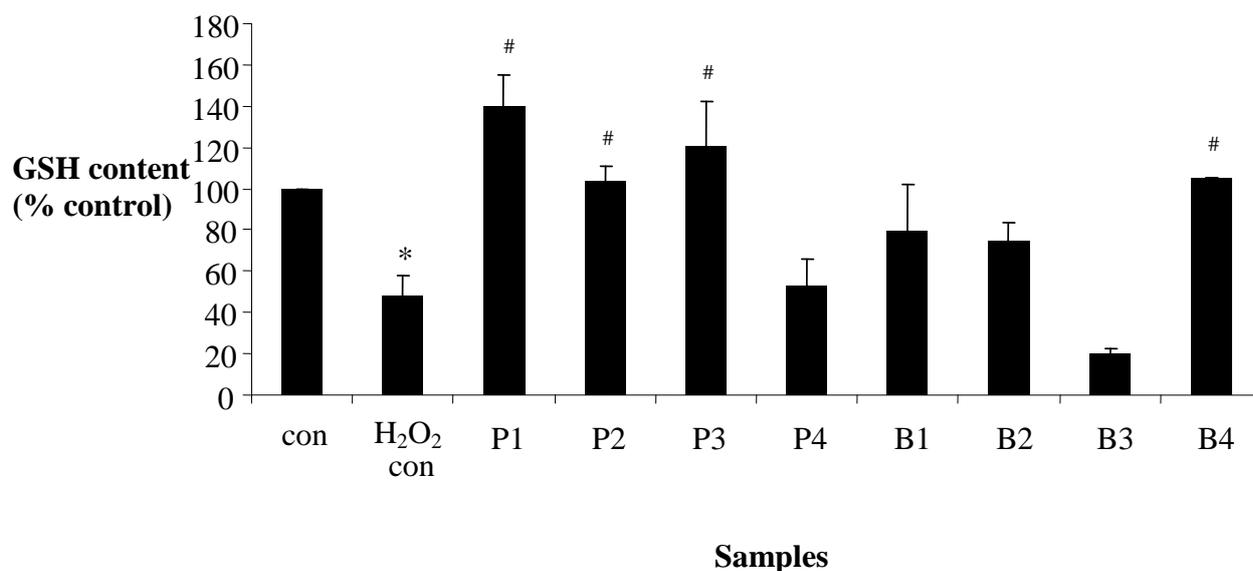


Figure 2: The antioxidant potential of pale (P) and black (B) brewers' spent grain (BSG) phenolic extracts, measured by their ability to protect against oxidative stress induced by hydrogen peroxide (H₂O₂) in the U937 cell line, using the glutathione (GSH) assay. Values represent the mean \pm SE of three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * Denotes statistically significant difference in GSH content between control and H₂O₂ control, $P < 0.05$. # Denotes statistically significant difference in GSH content between sample and H₂O₂ control, $P < 0.05$.

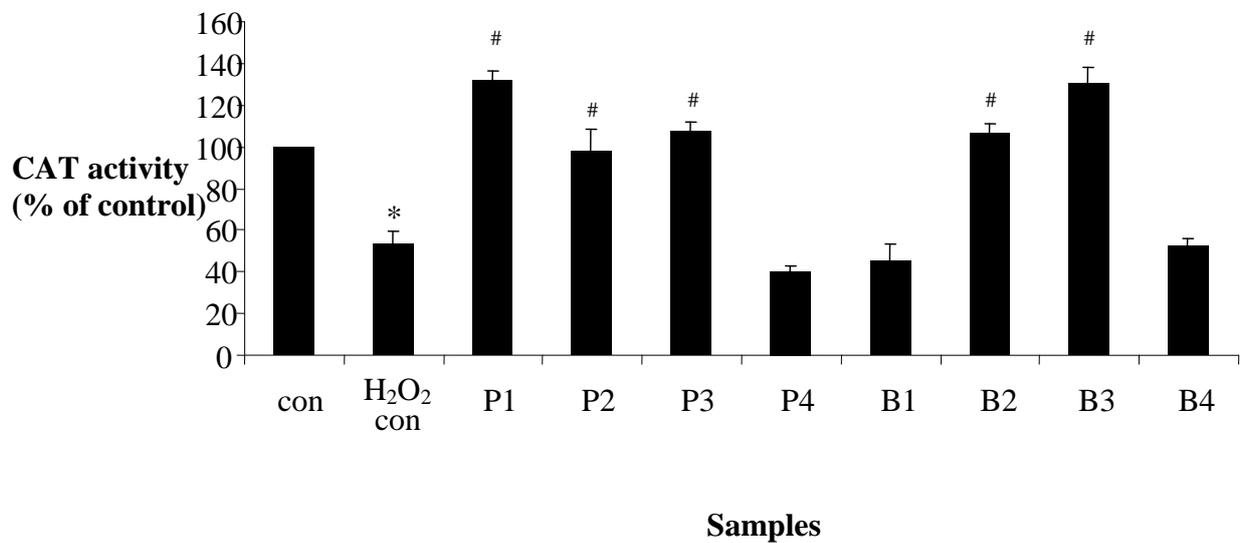


Figure 3: The antioxidant potential of pale (P) and black (B) brewers' spent grain (BSG) phenolic extracts, measured by their ability to protect against oxidative stress induced by hydrogen peroxide (H₂O₂) in the U937 cell line, using the catalase (CAT) assay. Values represent the mean \pm SE of three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * Denotes statistically significant difference in CAT activity between control and H₂O₂ control, $P < 0.01$. # Denotes statistically significant difference in CAT activity between sample and H₂O₂ control, $P < 0.05$.

Jurkat T cell line, with lower IC₅₀ values in this cell line compared to U937 cells (Table 3). Similarly, it has been reported that Jurkat T cells are more sensitive to epigallocatechin gallate (EGCG), while U937 cells are relatively more resistant to the cytotoxic effects of this polyphenol (Nakagawa *et al.*, 2004).

Inflammation has been associated with numerous human disorders including Parkinson's (Whitton, 2007) and Alzheimer's disease (Holmes *et al.*, 2009), in addition to conditions such as inflammatory bowel disease (Papadakis & Targan, 2000) and rheumatoid arthritis (Feldmann *et al.*, 1996). Given the prevalence and severity of these conditions, novel anti-inflammatory food-derived compounds could prove immensely beneficial.

Cytokines are cell-signalling molecules involved in the mediation and control of inflammatory and immune responses and can be categorised as either T helper 1 (Th1) or T helper 2 (Th2) cytokines. Th1 cytokines stimulate Type 1 cellular immunity, whereas Th2 cytokines play a role in Type 2 humoral immunity to produce immunoglobulins (Ig) (Spellberg & Edwards, 2001; Khajuria *et al.*, 2008). For the purposes of this study, cytokines were selected from both the Th1 (IL-2, IFN- γ) and Th2 (IL-4, IL-10) categories. BSG contains hydroxycinnamic acids, including ferulic, caffeic and *p*-coumaric acids, which have been reported to possess antioxidant (Kikuzaki *et al.*, 2002), anti-inflammatory (Hole *et al.*, 2012) and anti-atherogenic activity (Andreasen *et al.*, 2001). However, the immunomodulatory potential of phenolic extracts from BSG does not appear to have been studied.

This study showed that pale BSG phenolic extracts have greatest anti-inflammatory potential, with P2 significantly ($P < 0.05$) reducing IL-2, IL-4, IL-10 and IFN- γ production and P3 significantly ($P < 0.05$) reducing IL-2, IL-10 and IFN- γ production in ConA-stimulated Jurkat T cells (Table 4). Of the eight BSG phenolic extracts tested, P2 and P3 had highest hydroxycinnamic acid content (McCarthy *et al.*,

2013). It has previously been reported that hydroxycinnamic acids have anti-inflammatory effects, with cranberry and blueberry hydroxycinnamic acids protecting against the TNF- α stimulated production of inflammatory markers interleukin-8 (IL-8) and intercellular adhesion molecule (ICAM-1) (Youdim *et al.*, 2002). Derivatives of *p*-coumaric, ferulic and cinnamic acids isolated from corn bran inhibited nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) expression in lipopolysaccharide (LPS) induced RAW 264.7 macrophages (Kim *et al.*, 2012). *In vivo* animal studies also support findings for the anti-inflammatory effect of hydroxycinnamic acids, with *p*-coumaric acid reducing tumor necrosis factor (TNF- α) expression and circulating immune complex levels (Pragasam *et al.*, 2013). This anti-inflammatory potential suggests that the hydroxycinnamic acids present in extracts P2 (250.77 $\mu\text{g/mL}$) and P3 (139.34 $\mu\text{g/mL}$) may be responsible for their anti-inflammatory activity, as these are the extracts with highest hydroxycinnamic acid content.

IL-10 is an anti-inflammatory cytokine, which acts as an essential immunoregulator during infection (Couper *et al.*, 2008). It has been postulated that the strength of the regulatory response by IL-10 reflects the preceding inflammatory response (Couper *et al.*, 2008). Therefore, the measured reduction in IL-10 production in this study is thought to be indirect, and attributable to the phenolic extract-induced reduction in the inflammatory response.

Reactive oxygen species (ROS) have been linked to inflammation. For example, the generation of oxidants by silica results in increased expression of pro-inflammatory cytokines including TNF- α (Fubini & Hubbard, 2003). Furthermore, pretreatment of macrophages with antioxidants GSH or dimethyl sulfoxide (DMSO) prior to exposure to silica significantly decreased TNF- α production (Barrett *et al.*, 1999). Therefore, the ability of a compound to exert antioxidant activities could also protect against inflammation and have indirect anti-inflammatory effects.

In addition to inflammation, oxidative stress has been associated with metabolic syndrome and its clinical manifestations including hypertension, atherosclerosis and type 2 diabetes (Roberts & Sindhu, 2009), Parkinson's disease (Jenner, 2009) and Alzheimer's disease (Zawia *et al.*, 2009). Therefore, there is a continuing demand for novel anti-inflammatory compounds, which also possess antioxidant capabilities. This is particularly the case in regard to plant-derived compounds, which could be utilised as functional food ingredients. Peanut skin, a by-product of the peanut industry, has a low economic value and is mainly use as animal feed, similar to BSG (Yu *et al.*, 2005). Phenolic extracts from peanut skin have shown high antioxidant potential, greater than that of green tea infusions (Yu *et al.*, 2005). Other agro-industrial by-products such as olive mill waste (Obied *et al.*, 2007), fruit peel (Gorinstein *et al.*, 2002), potato peel waste (Sotillo *et al.*, 2006) and wine industry by-products (Makris *et al.*, 2007) have been suggested as inexpensive sources of bioactive compounds. BSG, a co-product of the brewing industry, has been incorporated into a range of foods including cookies (Prentice *et al.*, 1978; Ozturk *et al.*, 2002) and ready-to-eat snacks (Ainsworth *et al.*, 2007; Stojceska *et al.*, 2008) to increase the protein and fibre content.

Extract P1 had the greatest antioxidant effect and significantly protected against the H₂O₂-induced depletion in CAT and SOD activity and GSH content (Figure 1, 2 & 3). Extract B1 and P4 did not demonstrate any antioxidant effects under the conditions of the present study. The content of caffeic acid derivatives is lower in extract B1 compared to extract P1 (4.27 and 19.04 µg/mL, respectively; McCarthy *et al.*, 2013), which may account for the difference in antioxidant activity of P1 and B1. Recently, we reported that BSG phenolic extracts P2, P3 and B2, B3 have highest total phenolic content (TPC) and extract B4 has a higher TPC than extract P4 (McCarthy *et al.*, 2012). Therefore, the protection against oxidant-induced depletion of cellular antioxidants is speculated to be attributable to the TPC of the extracts, which is a measure of all reducing compounds in

the extract and not specifically the phenolic compounds (Huang *et al.*, 2005). Extracts B1 - B4 also significantly protected against H₂O₂-induced DNA damage in U937 cells (McCarthy *et al.*, 2012).

To conclude, the pale BSG extracts P2 and P3 possess anti-inflammatory properties, reducing stimulated cytokine production, which may be attributed to the hydroxycinnamic acid content of BSG extracts. Phenolic extracts P1 - P3 and B2 - B4 have the potential to protect against cellular oxidative stress induced by H₂O₂. BSG phenolic extracts represent a novel source of natural, plant-derived bioactive components with the potential to be exploited for the production of biofunctional foods for human consumption.

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Chapter 5

***In vitro* antioxidant and anti-inflammatory effects of brewers' spent grain protein rich isolate and its associated hydrolysates.**

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5.1. Abstract

Brewers' spent grain (BSG) is a protein-rich by-product of the brewing industry. The present study examined the *in vitro* bioactivity of a BSG protein enriched preparation and its associated enzymatic hydrolysates (assigned A - J). Cytotoxicity was measured using the (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT assay in U937 and Jurkat T cells. IC₅₀ values were lower in the U937 cell line, ranging from 4.93 to 9.27 % (v/v) versus a range of 4.11 % (v/v) to undetectable in Jurkat T cells. The superoxide dismutase (SOD) and comet assays were performed on U937 cells pre-incubated with test samples and subsequently exposed to an oxidant. Hydrogen peroxide (H₂O₂) significantly reduced SOD activity by 37.7 % and none of the test samples provided protection. None of the samples protected against DNA damage induced by *tert*-butylhydroperoxide (*t*-BOOH); hydrolysate H, prepared with Alcalase enzyme at 60 °C, protected against H₂O₂-induced DNA damage. The total phenolic content (TPC) was found to range from 0.021 to 0.055 mg GAE/mg dry powder. The effect of the BSG-derived test samples on cytokine production (IL-2, IL-4, IL-10, IFN- γ) in Concanavalin A (conA) stimulated Jurkat T cells was measured using an enzyme linked immunosorbent assay (ELISA). Samples had no effect on IL-2 and IL-4 production. The unhydrolysed sample C significantly reduced IL-10, while the protein rich isolate, unhydrolysed control samples and hydrolysates D, E, F, and J significantly reduced IFN- γ production. The BSG preparations possess little antioxidant potential and exhibit selective immunomodulatory effects that may be of benefit in the control of inflammatory diseases.

5.2. Introduction

Brewers' spent grain (BSG) is the residual solid fraction of barley malt remaining after wort is produced in the brewing process. Although BSG is mainly used as an animal feed, options for further uses are being explored. In recent years, there has been an

unprecedented and extensive demand for inexpensive plant-derived proteins and bioactive ingredients for human consumption. The protein content of BSG is approximately 20 % (w/dw) (Mussatto *et al.*, 2006), and this has potential to be exploited for human nutrition.

Protein hydrolysates have long been the focus of nutritional research for their ability to act as a source of biofunctional agents. Different protein hydrolysates have been shown to exert antioxidant and anti-inflammatory effects; these include pea protein (Ndiaye *et al.*, 2012), soy protein (Peña-Ramos & Xiong, 2002; Kong *et al.*, 2008), fish protein (Harnedy & FitzGerald, 2012) and casein hydrolysates (Phelan *et al.*, 2009; Lahart *et al.*, 2011). Protein hydrolysates have numerous uses in human nutrition ranging from protein supplementation of geriatric and sports nutrition products, energy drinks and weight-loss diets to clinical applications including treatment of Crohn's disease, liver disease and ulcerative colitis (Clemente, 2000). While much evidence exists with regard to protein hydrolysates from the aforementioned sources, to our knowledge, this is the first study on the bioactivity of protein preparations from BSG.

The antioxidant activity of a compound can be measured using an array of methods, both cellular and non-cellular. Superoxide dismutases are a group of metalloenzymes that catalyse the conversion of the superoxide anion ($O_2^{\cdot-}$) to molecular oxygen and hydrogen peroxide (H_2O_2), thus forming an essential part of the cellular antioxidant defence system. The single-cell gel electrophoresis assay (comet assay) is also used to determine antioxidant potential, through the induction of DNA damage (single-strand break) by an oxidant. In these assays, the antioxidant activity is determined by the ability of a compound to protect against oxidant-induced damage. Common oxidants employed in cellular antioxidant assays include *tert*-butylhydroperoxide (*t*-BOOH) and H_2O_2 (Aherne & O'Brien, 2000a; Aherne & O'Brien, 2000b; Alia *et al.*, 2005; Park *et al.*, 2003).

Cytokines are low molecular weight cell-signalling protein molecules including interleukins, interferons and tumor necrosis factor, which play a role in the inflammatory response. The ability of a compound to alter cytokine production may indicate anti-inflammatory potential and thus it may have beneficial effects for example, in inflammatory diseases such as atherosclerosis and rheumatoid arthritis. Jurkat T cells have been extensively used as a model for immunomodulatory studies (Phelan *et al.*, 2009; Benbernou *et al.*, 1997; Grassberger *et al.*, 1999).

The aims of the present study were: firstly, to assess the potential bioactivity of a protein rich isolate from BSG and its associated hydrolysates by measuring their cytotoxic and antioxidant effects in human monocytic blood cells (U937); secondly, to measure the ability of the BSG protein rich isolate/ hydrolysates to protect against oxidant-induced DNA damage in the U937 cell line; and thirdly, to determine potential immunomodulatory effects of the BSG protein isolate/ hydrolysates in concanavalin-A (con-A) stimulated human Jurkat T cells.

5.3. Materials and methods

5.3.1. Materials

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich Ireland Limited, Co. Wicklow, Ireland. U937 and Jurkat T cells were obtained from the European Collection of Animal Cell Cultures (ECACC).

5.3.2. Generation of protein rich isolate and protein hydrolysates

For production of the protein rich isolate (49.13 % protein dw), 940 g BSG was first sheared for 20 s at 11,000 rpm using an Ultra Turrax® T25 basic high performance disperser (IKA® Werke GmbH & Co. KG, Staufen, Germany) in 5.0 L dH₂O. The sheared samples were then allowed to stir gently for 1 hr at room temperature prior to

centrifugation at 2,700 g for 20 min at 10 °C (Hettich Zentrifugen Universal 320R centrifuge, Andreas Heitich GmbH & Co., Tuttlingen, Germany). The precipitate obtained from the above process was subjected to two sequential 1 hr extractions in 5.0 L 110 mM NaOH at 50 °C. The combined supernatants from the above extractions obtained after centrifugation were adjusted to pH 3.8, stirred gently for 15 min at room temperature and then centrifuged. The precipitated protein obtained was re-suspended in dH₂O, neutralised to pH 7.0 using 2 N NaOH and freeze-dried.

The freeze dried protein rich isolate (49.13 % protein dw) was hydrolysed with the commercially available enzymes Corolase PP, Flavourzyme and Alcalase 2.4L under conditions described in Table 1. The pH was maintained by addition of 0.5 N 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 hydrolysates were each adjusted to pH 7.0, heated at 95 °C for 10 min to inactivate the enzyme and were then freeze dried. Prior to analysis, freeze-dried samples were made up to 1 % (w/v) solution with distilled deionised water, filter sterilised, aliquoted into sterile eppendorphs and stored at -20 °C.

5.3.3. Total phenolic content (TPC)

The total phenolic content (TPC) of the protein rich isolate and its associated hydrolysates was measured using the Folin-Ciocalteu method as described previously (Singleton & Rossi, 1965). This assay measures the ability of a compound to reduce the yellow oxidising Folin-Ciocalteu reagent to a blue/green colour. Absorbance is measured spectrophotometrically at 765 nm and results are expressed as mg gallic acid equivalents per mg dry powder (mg GAE/mg dry powder).

Table 1: Preparation of protein isolate/hydrolysate test samples from brewers' spent grain (BSG).

Sample	Description	Enzyme/water	E/S Ratio	Temperature (°C)	pH	Time (h)
A	BSG protein rich isolate	-	-	-	-	-
B	Control for D, E, F, G, J	water	-	50	7	4
C	Control for H, I	water	-	60	9	4
D	BSG protein hydrolysate	Corolase PP	1% (w/w)	50	7	4
E	BSG protein hydrolysate	Corolase PP	2.5% (w/w)	50	7	4
F	BSG protein hydrolysate	Flavourzyme	1% (v/w)	50	7	4
G	BSG protein hydrolysate	Flavourzyme	2.5% (v/w)	50	7	4
H	BSG protein hydrolysate	Alcalase 2.4L	1% (v/w)	60	9	4
I	BSG protein hydrolysate	Alcalase 2.4L	2.5% (v/w)	60	9	4
J	BSG protein hydrolysate	Alcalase 2.4L	2.5% (v/w)	50	7	4

5.3.4. Cell culture

U937 cells, a human monocytic blood cell line, and Jurkat T cells, a human leukaemic T cell line, were maintained in antibiotic-free RPMI-1640 medium supplemented with 10 % (v/v) fetal bovine serum (FBS), at 37 °C in a 5 % CO₂ atmosphere. Cell densities of 1 x 10⁵ cells/mL (cytotoxicity and superoxide dismutase assays) or 2 x 10⁵ cells/mL (comet assay and enzyme-linked immunosorbent assays) were used for experimentation. Reduced serum media (2.5 % FBS) was used for all experiments.

5.3.5. Cell proliferation

To measure cell proliferation the (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was carried out in both the U937 and Jurkat T cell lines. Cells (1 x 10⁵ cells/mL) were incubated for 24 hr at 37 °C with test samples (A - J) at concentrations ranging from 0 - 20 % (v/v). To account for the colour of the samples, a media blank was also included (samples and media only, no cells). The MTT assay kit was then used to quantify cell proliferation. In this assay, MTT (a yellow tetrazolium salt) is converted to a formazan derivative (purple colour), and the absorbance of the converted dye is measured at 570 nm. Briefly, 10 µL MTT reagent 1 and 100 µL RPMI was added to cells and incubated for 4 hr at 37 °C. Following incubation, 100 µL was removed from the wells and 100 µL MTT reagent 2 was added. A further incubation period of 24 hr at 37 °C followed. Absorbance was read at 570 nm using a microplate reader (Spectrafluorplus, Tecan). Using cell proliferation data from the MTT assay, IC₅₀ values (the concentration of a test sample that induces 50 % cell death) were determined. GraphPad Prism 4 was used for analysis and IC₅₀ values are expressed as % (v/v). On the basis of the MTT assay and published literature (Cumby *et al.*, 2008; Lahart *et al.*, 2011; Piccolomini *et al.*,

2012), a non-toxic concentration of 0.5 % (v/v) BSG protein preparations was used for further analyses.

5.3.6. Superoxide dismutase (SOD) assay

U937 cells (5 mL) were seeded at a density of 1×10^5 cells/mL in T25 flasks. Cells were incubated for 24 hr with 0.5 % (v/v) test sample and subsequently treated with 100 μ M H_2O_2 for 60 min. SOD activity was measured using a Calbiochem SOD Assay Kit II (Merck Chemicals Ltd., Nottingham, U.K.) The protein content of the BSG protein samples was measured using the Bicinchoninic acid (BCA) protein assay, as previously described (Smith *et al.*, 1985). The SOD activity is expressed relative to the protein content, as SOD units/mg protein in cell homogenate ($U\ mg^{-1}$ protein). One SOD unit is defined as the amount of enzyme needed to exhibit 50 % dismutation of the superoxide radical.

5.3.7. Comet assay

U937 cells (1×10^5 cells/mL) were treated with 0.5 % (v/v) protein preparations for 24 hr in a 6-well plate (final volume 2 mL) at 37 °C. Following incubation, cells were treated with 50 μ M H_2O_2 for 30 min or 400 μ M *t*-BOOH for 60 min. Cell viability was then determined using the fluorescein diacetate ethidium bromide (FDA/EtBr) assay. The comet assay was used to measure oxidative DNA damage in the U937 cells, as previously described by McCarthy *et al.* (2012). Briefly, cells were harvested and embedded on microscope slides using low melting point (LMP) agarose. Lysis was carried out for 1 hr at 4 °C, before electrophoresis for 25 min at 21 V, 300 mA. The slides were neutralised using 0.4 M Tris and stained with 20 μ g/mL EtBr. Comet 5.5 image analysis software was used to score 50 cells for each slide using a fluorescence microscope (Optiphot-2, Nikon). DNA damage was expressed as percentage tail DNA.

5.3.8. Cytokine production

Jurkat cells, at a density of 2×10^5 cells/mL, were seeded in 96-well plates in the presence of ConcanavalinA (conA, 50 μ g/mL) and treated with 0.5 % (v/v) test samples for 24 hr. Production of the cytokines interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-10 (IL-10) and interferon- γ (IFN- γ) was determined using eBioscience ELISA kits (Human Th1/Th2 ELISA Ready-SET-Go kit purchased from Insight Biotechnology, Wembley, UK). Absorbance was read at 450 nm using a microplate reader (ThermoScientific Varioskan Flash).

5.3.9. Statistical analysis

All data represents the mean and standard error values of at least three independent experiments. Data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett's test (or Tukey's multiple comparison test, where appropriate), using GraphPad Prism 4 (GraphPad software, California, U.S.A.).

5.4. Results

Ten samples (A - J) were analysed during the course of the study. These included the BSG protein rich isolate (A), control at 50 °C (B), control at 60 °C (C) and seven protein hydrolysates (D - J).

Molecular weight distribution profiles (GP-HPLC) of hydrolysates show that up to 80 % of the peptides were < 5 kDa when compared to the unhydrolysed BSG protein rich isolate which had 32 % of proteins and peptides < 5 kDa. Of the three enzymes, hydrolysates generated with Flavourzyme had the lowest percentage of proteins and peptides < 5 kDa with values of 64 and 52 % for the high and low E/S ratio, respectively.

5.4.1. Total phenolic content (TPC)

The TPC of the protein/hydrolysate preparations was found to range from 0.021 to 0.055 mg GAE/mg dry powder (Table 2). Hydrolysate H prepared with Alcalase at 60 °C had a significantly ($P < 0.01$) higher TPC than its corresponding control (C). Compared with the protein rich isolate A, hydrolysates H and J (also prepared with Alcalase) had significantly ($P < 0.05$) higher TPC.

5.4.2. Cell proliferation

A range of concentrations (0 - 20 % v/v) of test samples were incubated at 37 °C for 24 hr with either U937 or Jurkat T cells in 96-well plates, the MTT assay was carried out and absorbance (which is related to the percentage cell proliferation) was read at 570 nm. As shown in Table 3, all samples A - J reduced U937 cell proliferation in a dose-dependent manner. Concentrations of 8, 10 and 20 % (v/v) of all samples (except E) significantly ($P < 0.05$) reduced U937 cell viability. Using GraphPad Prism 4, the IC_{50} values of each sample were computed (Table 3) and found to range from 4.93 to 9.27 % (v/v). For further experimentation, a non-toxic concentration of 0.5 % (v/v) protein sample was selected. In the Jurkat T cell line, it was found that samples A - H stimulated growth; with each of these samples significantly increasing cell viability at 2, 2.5 and 4 % (v/v) addition (Table 4). In comparison, hydrolysates I and J, prepared with Alcalase at 60 °C and 50 °C, respectively, significantly reduced cell viability in a dose-dependent manner (Table 4). Similar to the trend observed in the U937 cell line, sample I significantly ($P < 0.05$) reduced cell viability at concentrations greater than 4 % (v/v). The IC_{50} values in the Jurkat T cells for samples I and J were lower than that for the U937 cell line (Table 4), IC_{50} values for other samples could not be determined. For immunomodulatory assays using the Jurkat T cells, the 0.5 % (v/v) level of addition of BSG test samples was chosen, as this level did not cause significant changes in cell viability across all samples.

Table 2: Total phenolic content (TPC) of protein isolate and associated hydrolysates from brewers' spent grain (BSG).

Test sample ^a	TPC content (mg gallic acid equivalents (GAE)/mg dry powder)
A	0.029 ± 0.003
B	0.043 ± 0.004
C	0.021 ± 0.002
D	0.039 ± 0.003
E	0.034 ± 0.001
F	0.032 ± 0.001
G	0.046 ± 0.002
H	0.049 ± 0.004* [#]
I	0.033 ± 0.002
J	0.055 ± 0.006*

^a A = unhydrolysed starting material, B = control at 50 °C, C = control at 60 °C, D – J = protein hydrolysates of BSG. Data represents the mean ± SE of three independent experiments. Statistical analysis by ANOVA followed by Tukey's multiple comparison test. * Statistically significant difference between protein rich isolate A and hydrolysate (P < 0.05). [#] Statistically significant difference between relevant control and corresponding hydrolysate (P < 0.01).

Table 3: Effect of BSG protein hydrolysates (0 – 20 % v/v) on cell proliferation in the U937 cell line.

Sample ^a	Cell proliferation (% of control) ^b											IC ₅₀ ^c (% v/v)
	0%	0.5%	1%	2%	2.5%	4%	5%	6%	8%	10%	20%	
A	100.00	83.06	84.35	80.56	78.26	77.94	75.44	67.68	39.78*	9.80*	1.95*	7.80
B	100.00	76.98	88.22	97.48	97.38	95.74	105.61	81.42	34.21*	23.90*	-0.16*	7.43
C	100.00	72.29	71.68	80.50	98.47	98.03	78.23	77.34	39.45*	47.45*	3.11*	9.27
D	100.00	96.73	100.38	102.97	101.79	93.18	109.61	99.84	46.84*	8.60*	9.56*	7.92
E	100.00	80.38	87.55	92.74	93.60	86.10	82.12	46.00	22.13	7.10	11.06	5.95
F	100.00	87.49	87.90	87.90	93.86	93.94	114.20	89.47	67.23*	18.38*	16.05*	8.31
G	100.00	95.23	96.06	89.91	94.63	102.66	98.10	91.41	58.44*	10.60*	25.30*	8.01
H	100.00	53.63	72.63	80.82	90.69	86.40	68.86*	42.11*	4.53*	1.82*	6.36*	6.04
I	100.00	73.98*	70.94	93.88	90.91	57.58*	41.93*	33.88*	3.80*	6.12*	11.47*	4.93
J	100.00	77.06*	61.62	88.43	92.07	87.88	85.49	81.04	61.58*	37.39*	33.17*	8.21

^a A = unhydrolysed starting material, B = control at 50 °C, C = control at 60 °C, D – J = protein hydrolysates of BSG ^b Values are mean of four independent experiments, expressed as a percentage relative to untreated U937 cells. ^c Data values relate to the amount of protein hydrolysate added to cells, expressed as % (v/v). IC₅₀ values were determined using cell proliferation data from the MTT assay and GraphPad Prism, version 4.00. * Denotes statistically significant difference in cell viability, relative to untreated U937 cells (P < 0.05), by ANOVA followed by Dunnett's Test.

Table 4: Effect of BSG protein hydrolysates (0 – 20 % v/v) on cell proliferation in the Jurkat T cell line.

Sample ^a	Cell proliferation (% of control) ^b											IC ₅₀ ^c (% v/v)
	0%	0.5%	1%	2%	2.5%	4%	5%	6%	8%	10%	20%	
A	100.00	95.40	81.40	207.63*	203.86*	189.35*	181.30*	163.25	151.71	139.51	153.92	nd
B	100.00	82.26	92.20	202.11*	202.50*	188.00*	218.59*	162.62	160.40	139.17	159.30	nd
C	100.00	90.95	95.73	203.25*	233.40*	195.45*	202.66*	152.11	145.32	139.58	174.33*	nd
D	100.00	94.56	88.46	197.62*	206.29*	210.93*	200.81*	166.80*	154.48	141.80	147.18	nd
E	100.00	85.59	98.68	224.05*	214.41*	184.61*	165.54*	132.94	132.30	142.09	123.07	nd
F	100.00	75.17	191.23*	202.93*	217.00*	217.59*	184.56*	175.57*	134.81	140.36	140.28	nd
G	100.00	95.34	169.61	169.60	217.39*	223.19*	185.43*	186.90*	136.85	151.28	142.66	nd
H	100.00	82.13	183.29*	182.15*	217.14*	201.52*	152.34	155.24	120.11	114.02	103.30	nd
I	100.00	74.15	76.31	79.56	75.74	50.05*	16.15*	15.71*	13.53*	26.91*	-6.30*	4.11
J	100.00	83.89	84.79	90.25	88.38	65.66	40.20*	16.29*	34.90*	27.55*	1.00*	4.43

^a A = unhydrolysed starting material, B = control at 50 °C, C = control at 60 °C, D - J= protein hydrolysates of BSG ^b Values are mean of four independent experiments, expressed as a percentage relative to untreated Jurkat T cells ^c data values relate to the amount of protein hydrolysate added to cells, expressed as % (v/v). IC₅₀ values were determined using cell proliferation data from the MTT assay and GraphPad Prism, version 4.00. * Denotes statistically significant difference in cell viability, relative to untreated Jurkat T cells (P < 0.05), by ANOVA followed by Dunnett's Test.

5.4.3. Antioxidant activity

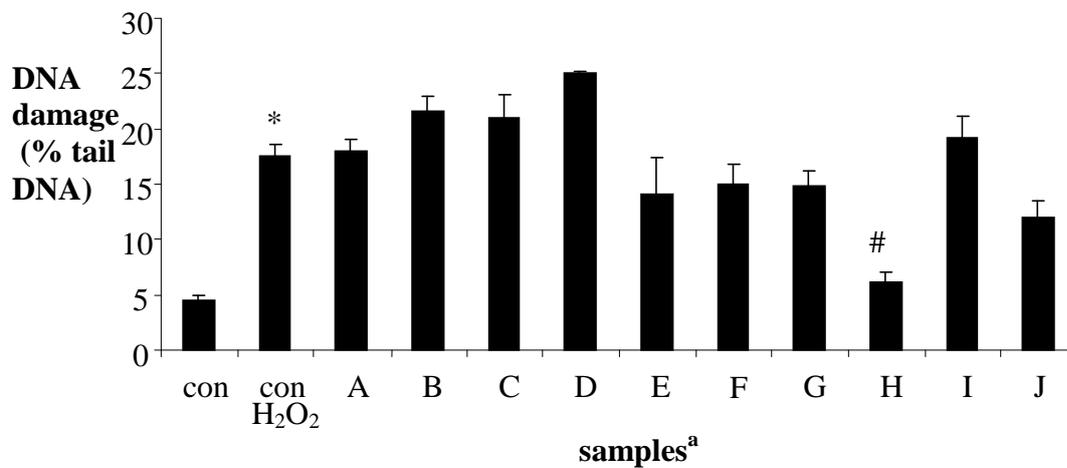
Two cellular assays were employed to assess the antioxidant potential of the BSG protein samples – the SOD assay and the comet assay. For the SOD assay, oxidative stress was induced in U937 cells with 100 μM H_2O_2 and the ability of the test samples to protect against this oxidation was measured (Table 5). H_2O_2 significantly reduced SOD activity (expressed as SOD units (U)/mg protein) from 0.20 ± 0.01 to 0.12 ± 0.02 . Protein rich isolate (A) did not change the % SOD activity, compared to the H_2O_2 control. Control samples B and C increased the % SOD activity by 12.6 and 22.7 %, respectively. None of the protein hydrolysates (D - J) provided any significant change from the H_2O_2 control value. Compared to the unhydrolysed starting material (A), hydrolysates D, G, I and J (all prepared at 50 °C with Corolase, Flavourzyme or Alcalase, respectively) increased the % SOD activity by 14, 13, 9 and 24 %, respectively. Hydrolysate J increased the % SOD activity by 12 % compared to its corresponding control preparation B. Hydrolysates H and I were less antioxidant than their corresponding control (C) in terms of relative SOD activity. The ability of the test samples to protect against oxidant induced DNA damage was assessed using the comet assay. Both oxidants (H_2O_2 and *t*-BOOH) significantly increased the percentage DNA damage (expressed as % tail DNA); none of the test samples provided significant protection against these increases in tail DNA, with the exception of hydrolysate H which significantly protected against H_2O_2 -induced DNA damage (Figure 1a). In cells treated with both H_2O_2 and hydrolysates E, F, G, H and J there was less DNA damage than in cells treated with control preparations A, B and C. Hydrolysates G, H, I and J provided greater protection than unhydrolysed protein preparations A, B and C against DNA damage induced by *t*-BOOH (Figure 1b).

Table 5: Ability of brewers' spent grain (BSG) protein isolate and associated hydrolysates (0.5 % v/v) to protect against oxidative stress induced by hydrogen peroxide (H₂O₂) in the U937 cell line, using the superoxide dismutase (SOD) assay.

Test sample ^a	SOD activity (U mg ⁻¹ protein)	SOD activity (% of control)
Control	0.20 ± 0.01	100.00
H ₂ O ₂ control	0.12 ± 0.02*	62.35 *
A	0.12 ± 0.02	63.04
B	0.15 ± 0.02	74.92
C	0.17 ± 0.02	85.00
D	0.15 ± 0.04	77.20
E	0.13 ± 0.01	66.25
F	0.12 ± 0.02	66.22
G	0.15 ± 0.04	76.71
H	0.13 ± 0.02	65.87
I	0.14 ± 0.02	72.03
J	0.17 ± 0.04	87.20

BSG, brewers' spent grain; H₂O₂, hydrogen peroxide; SOD, superoxide dismutase. ^a A = unhydrolysed starting material, B = control at 50 °C, C = control at 60 °C, D – J = protein hydrolysates of BSG. Data represents the mean (± SE) of five independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * Indicates statistically significant difference in SOD activity between control and H₂O₂ control (P < 0.01).

(a)



(b)

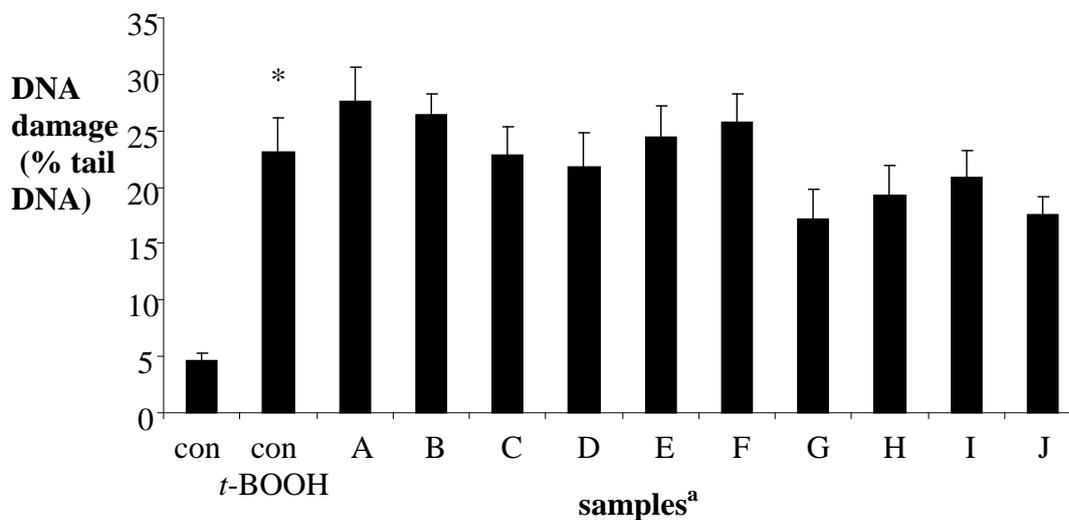


Figure 1: The ability of BSG protein hydrolysates to protect against DNA damage induced by (a) H₂O₂ (b) *t*-BOOH. ^a A = unhydrolysed starting material, B = control at 50 °C, C= control at 60 °C, D – J = protein hydrolysates of BSG. Values are mean ± SE of five independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * Indicates a statistically significant difference in DNA damage between control and oxidant control (P < 0.01). # Indicates statistically significant difference in DNA damage, relative to H₂O₂ control (P < 0.01).

5.4.4. Immunomodulatory activity

BSG protein hydrolysates were assessed for their immunomodulatory activity. Following stimulation of Jurkat T cells with 50 µg/mL conA, cytokine production was measured (IL-2, IL-4, IL-10, IFN-γ), with results shown in Table 6. There was no significant change in IL-2 or IL-4 production following supplementation with any of the test samples. IL-10 production was unaffected by the hydrolysates. Protein rich isolate control sample C significantly reduced IL-10 production ($P < 0.05$). A more marked effect was observed for IFN-γ production, with all unhydrolysed isolates (A, B and C) and all hydrolysates except G, H and I significantly reducing IFN-γ production; these samples gave a minimum reduction of 20 %. As was also seen in the SOD assay results, hydrolysates H and I prepared with Alcalase at 60 °C are less bioactive than their unhydrolysed control preparation (C) by the ELISA assay, having no effect on IL-10 production in conA stimulated Jurkat T cells. It was found that the unhydrolysed starting material (A) had more of an anti-inflammatory effect than F, G, H and I for all cytokines measured (Table 6). Hydrolysate J, which had the highest TPC and SOD activity reduced IL-2 and IL-10 to the greatest extent and was the second most active hydrolysate at reducing IL-4 and IFN- γ production.

5.5. Discussion

Due to its low commercial value and high nutritional value, in addition to the vast quantities produced, BSG is an attractive co-product of the brewing industry. Current use is limited to animal feed and alternative uses of BSG are highly sought after. The antioxidant potential of phenolic extracts from BSG has been previously reported (McCarthy *et al.*, 2012); the focus of the present study was on the bioactive potential of a BSG protein rich isolate and its associated hydrolysates. As already outlined, the protein rich isolate from BSG contained 49.13 % protein as determined by Kjeldahl N

Table 6: The effect of protein isolate and hydrolysates from brewer's spent grain (0.5 % v/v) on cytokine production in concanavalin-A stimulated Jurkat T cells.

BSG sample ^a	Cytokine Production			
	(% of control)			
	IL-2	IL-4	IL-10	IFN- γ
Control	100.00 \pm 0.00	100.00 \pm 0.00	100.00 \pm 0.00	100.00 \pm 0.00
A	87.77 \pm 6.87	86.31 \pm 3.49	92.02 \pm 2.06	73.38 \pm 4.59*
B	78.62 \pm 5.40	98.44 \pm 10.54	87.57 \pm 2.33	67.99 \pm 2.17*
C	84.26 \pm 7.56	91.64 \pm 3.46	85.21 \pm 5.25*	76.31 \pm 6.10*
D	95.11 \pm 9.34	95.13 \pm 2.82	91.50 \pm 2.59	78.98 \pm 7.10 [#]
E	89.74 \pm 10.64	89.54 \pm 2.18	92.40 \pm 4.26	73.22 \pm 2.98*
F	103.97 \pm 14.37	102.67 \pm 3.88	94.78 \pm 3.05	80.16 \pm 5.72 [#]
G	93.73 \pm 9.49	99.86 \pm 1.33	97.42 \pm 3.76	81.89 \pm 3.42
H	107.97 \pm 14.75	115.17 \pm 7.88	95.61 \pm 0.79	85.86 \pm 4.26
I	96.50 \pm 10.91	95.62 \pm 3.81	103.44 \pm 1.64 [†]	84.31 \pm 3.27
J	81.74 \pm 1.74	94.80 \pm 4.84	88.41 \pm 3.33	78.86 \pm 5.03 [#]

BSG, brewers' spent grain. ^a A = unhydrolysed starting material, B = control at 50 °C, C = control at 60 °C, D – J = protein hydrolysates of BSG ^b Values are mean \pm SE of at least three independent experiments, expressed as a percentage relative to cells treated with conA alone. Statistical analysis by ANOVA followed by Dunnett's test. * P < 0.01 [#] P < 0.05, statistically significant difference in cytokine production between control and cells treated with protein hydrolysate. [†] Denotes statistically significant difference (P < 0.05) between hydrolysate I and corresponding control C.

determination (Connolly *et al.*, 2013). Interestingly, heat treatment of the unhydrolysed BSG protein isolate appeared to differentially affect the TPC. The unhydrolysed sample heated at 50 °C had higher mean TPC (0.043) in comparison to the unheated and sample heated at 60 °C (0.029 and 0.021 mg GAE/mg dry powder, respectively). Furthermore, hydrolysate samples H and J had TPC samples of 0.049 and 0.055 mg GAE/mg dry powder, respectively. This would seem to indicate that the specific hydrolysis process used to generate those samples enhanced TPC activity. It has been suggested in the literature that a number of factors can influence the polyphenol concentration following hydrolysis. These include polyphenol-protein interactions and bonding, type of enzyme used for extraction and the release of simple sugars and oligosaccharides during cell wall polysaccharide degradation, resulting in lower polyphenol content of extracts (Wang *et al.*, 2010).

The effect of the test samples on cell proliferation was assessed using the MTT assay. In addition to screening the hydrolysates for their cytotoxic effects, this assay also provided information regarding the concentration of test samples to be used for further analysis in the antioxidant and immunomodulatory assays. The IC₅₀ values (as % v/v) calculated for the BSG test samples I and J were lower than those observed for casein hydrolysates in the Jurkat cell line (Phelan *et al.*, 2009). This study also showed that the IC₅₀ value of some hydrolysates could not be determined, indicating a high tolerance of these cells to supplementation with the test samples.

SOD represents the first line of enzymatic antioxidant defence in the body, catalysing the conversion of the superoxide anion to hydrogen peroxide, which then undergoes detoxification by glutathione peroxidase and catalase (Mittler, 2002). H₂O₂-induced reduction in SOD activity, as shown here, has also been reported in the literature at varying H₂O₂ concentrations in a range of cell lines (Zhai *et al.*, 2011; O'Sullivan *et al.*, 2011; Zhong *et al.*, 2011). It has been proposed that H₂O₂ exerts its effect on SOD by

inactivating the enzyme (Salo *et al.*, 1988). More specifically, H_2O_2 causes a reduction of Cu^{2+} to Cu^+ , and subsequently reacts with the Cu^+ to form $Cu^{2+}OH^-$, which has the potential to oxidatively damage a histidine and thus inactivate the SOD enzyme (Hodgson & Fridovich, 1975).

For this study, two oxidants were utilised to induce DNA damage, H_2O_2 and *t*-BOOH. These oxidants were chosen on the basis that they exert their oxidative effects by different mechanisms. While H_2O_2 induces oxidative stress in an iron-dependent manner, by means of the Fenton reaction (Imlay *et al.*, 1988), iron has a minor effect on cell susceptibility to *t*-BOOH induced oxidative stress (Kruszewski *et al.*, 2008).

In general, test samples from BSG did not exhibit significant antioxidant activity by either the SOD activity or the comet assays (except hydrolysate H). Comparing the SOD and TPC assay results, hydrolysate J had highest TPC (0.055 mg GAE/mg dry powder) and also showed greatest protection against oxidation induced by H_2O_2 . This relationship between TPC and antioxidant activity has been previously reported (McCarthy *et al.*, 2012).

The data on these novel BSG-derived samples can be compared to existing literature regarding protein hydrolysates from a range of other sources. It has been shown that casein hydrolysates do not protect against H_2O_2 -induced DNA damage (Phelan *et al.*, 2009). In contrast, there is some evidence to suggest that protein hydrolysates from other sources can protect against oxidative stress induced by H_2O_2 , for example, tuna liver protein (Je *et al.*, 2009) and algae protein hydrolysates (Sheih *et al.*, 2009).

Cytokines, cell-signalling protein molecules, are involved in the inflammatory response. The excessive production of cytokines has been linked to a number of physiological conditions including inflammatory bowel disease (Papadakis & Targan, 2000) and rheumatoid arthritis (Feldmann *et al.*, 1996). Similarly, insufficient production of cytokines can play a role in immunodeficient disease states. Immunity can be classed

as either Type 1 or Type 2. Type 1 immunity is stimulated in T helper 1 (Th1) lymphocytes by cytokines including IL-2, IFN- γ (Spellberg & Edwards, 2001) and can be regarded as cellular immunity (Khajuria *et al.*, 2008). IL-4, IL-5, IL-9, IL-10 play a role in type 2 immunity stimulated in T helper 2 (Th2) lymphocytes (Spellberg & Edwards, 2001). Type 2 immunity is humoral immunity and Th2 cells support the production of immunoglobulins (Ig) including IgA and IgM (Khajuria *et al.*, 2008). ConA is a T cell lectin mitogen, that stimulates the production of cytokines by activation of the mitogen-activated protein kinase (MAPK) via the T-cell receptor (Tanaka *et al.*, 2005). Jurkat T lymphocytes stimulated with conA have previously been used as a model for assessing the immunomodulatory effects of different compounds (Aherne & O'Brien, 2008; Verlengia *et al.*, 2004).

Much evidence exists regarding the immunomodulatory potential of protein hydrolysates from a range of sources, although protein hydrolysates from BSG have not been studied to date. In 2008, it was reported that a protein hydrolysate from the seaweed, *Ecklonia cava*, reduced the conA stimulated production of IFN γ - and TNF- α in splenocytes (Ahn *et al.*, 2008). Hydrolysates from other protein sources also display similar immunomodulatory activity. One such source is soy protein, and specifically the lunasin peptide isolated from soy. Lunasin has been shown to reduce the production of a number of inflammatory markers including TNF- α , IL-6, nitric oxide (NO) and prostaglandin E₂ (PGE₂) (Hernandez-Ledesma *et al.*, 2009; Dia *et al.*, 2009). Protein isolated from sources such as whey protein (Cross & Gill, 1999), flaxseed meal (Udenigwe *et al.*, 2009) and yak milk casein (Mao *et al.*, 2011) also exhibit anti-inflammatory potential. Results of the present study suggest that BSG protein isolate and associated unhydrolysed samples and hydrolysates selectively reduce production of cytokines. Further investigation may yield hydrolysates with greater bioactivity, similar

to that observed for the aforementioned hydrolysates from whey (Cross & Gill, 2009), flaxseed (Udenigwe *et al.*, 2009) and yak milk casein (Mao *et al.*, 2011).

IFN- γ is considered a pro-inflammatory cytokine, as it enhances NO production and TNF activity and is effective at stimulating pro-inflammatory gene expression such as inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) (Dinarello, 2000). IL-1, TNF- α and IFN- γ can initiate an inflammatory cascade as shown in Figure 2. The beneficial effect of the test samples is further supported by the theory that IL-4 and IL-10 are anti-inflammatory cytokines (Isomaki & Punnonen, 1997). BSG protein hydrolysates did not significantly reduce the production of these cytokines, but selectively reduced pro-inflammatory IFN- γ production, as did the protein rich isolates from which the hydrolysates were prepared. Hydrolysis of the proteins did not enhance their immunomodulatory effects. It has been reported that IL-10 acts as a potent inhibitor of IL-2 and IFN- γ , making it the most important anti-inflammatory cytokine. This has led to its use in clinical trials for the treatment of conditions such as inflammatory bowel disease (Opal & DePalo, 2000). Recent treatments for anti-inflammatory diseases such as rheumatoid arthritis (Dinarello, 2000), juvenile idiopathic arthritis and Crohn's disease (Nishimoto & Kishimoto, 2004) are centered around increasing anti-inflammatory and inhibiting pro-inflammatory cytokines. Therefore, the ability of a compound to selectively inhibit pro-inflammatory cytokine production shows great potential for the nutraceutical industry, for the treatment of inflammatory diseases. It is important to note that while the protein rich isolate contains approximately 50 % protein, there is a possibility that components of the remaining 50 % (consisting primarily of carbohydrate) may account for the bioactivity of the BSG protein isolate and associated hydrolysates.

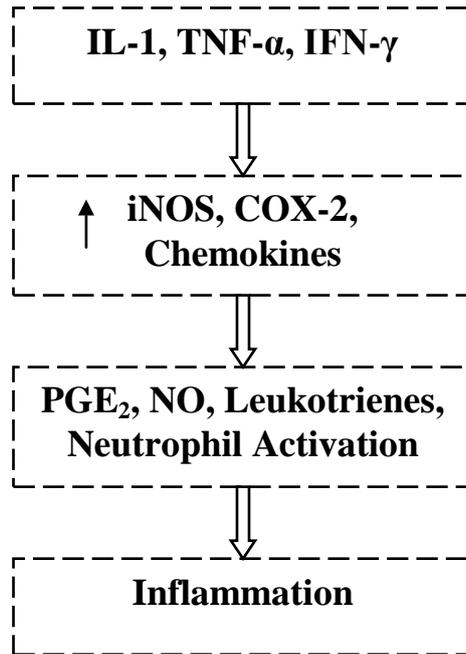


Figure 2: The inflammatory cascade, as initiated by IL-1, TNF- α and IFN- γ . IL-1: interleukin 1, TNF- α : tumor necrosis factor alpha, IFN- γ ; interferon gamma, iNOS: inducible nitric oxide synthase, COX-2: cyclooxygenase 2, PGE₂: prostaglandin E2, NO: nitric oxide. IL-1, TNF- α and IFN- γ stimulates increased expression of pro-inflammatory genes such as iNOS and COX-2 and synthesis of chemokines. This leads to the production of leukotrienes, NO and PGE₂ and the activation of neutrophils, resulting in inflammation, tissue damage and loss of function.

5.6. Conclusion

The findings of the present study suggest that protein preparations from BSG do not show antioxidant potential, but may have selective immunomodulatory effects. It is possible that the presence of phenolic compounds in the preparations plays a role in the observed effects. The ability of the BSG protein isolate and associated hydrolysates to significantly decrease pro-inflammatory cytokine IFN- γ production may prove useful in the treatment of inflammatory diseases. These data warrant the further investigation of BSG protein rich isolate and associated hydrolysates for their immunomodulatory effects.

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Chapter 6

Brewers' spent grain (BSG) protein hydrolysates decrease hydrogen peroxide (H₂O₂)-induced oxidative stress and concanavalin-A (con-A) stimulated IFN- γ production in cell culture.

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6.1. Abstract

The present study investigated the bioactivity of protein hydrolysates and fractionated hydrolysates prepared from brewers' spent grain (BSG) using proteases, including Alcalase 2.4L, Flavourzyme and Corolase PP. Hydrolysates were designated K-Y, including fractionated hydrolysates with molecular weight (m.w.) < 3, < 5 and > 5 kDa. Where computable, IC₅₀ values were lower in U937 (1.38 % - 9.78 %) than Jurkat T cells (1.15 % - 13.82 %). Hydrolysates L, Q and R and fractionated hydrolysates of U and W (< 3, < 5, > 5 kDa) significantly (P < 0.01) protected against hydrogen peroxide (H₂O₂)-induced reduction of superoxide dismutase (SOD) activity. A fractionated hydrolysate of W (< 5 kDa) protected against H₂O₂-induced DNA damage, P < 0.01. Hydrolysates K, N, P, U, U > 5 kDa, V, V > 5 kDa, W, W > 5 kDa significantly (P < 0.05) reduced a concanavalin-A (con-A) stimulated production of interferon- γ (IFN- γ). In conclusion, BSG protein hydrolysates demonstrate bioactivity *in vitro*; lower m.w. hydrolysates (< 3, < 5 kDa) show greatest antioxidant activity and unfractionated or higher m.w. hydrolysates (> 5 kDa) possess anti-inflammatory effects.

6.2. Introduction

Brewers' spent grain (BSG) represents 85 % of the total solid by-products of the brewing industry, with 8.5 million tons of dry BSG produced on an annual basis globally (Xiros & Christakopoulos, 2012). This lignocellulosic material is an excellent source of protein and fibre in addition to polyphenolic compounds (hydroxycinnamic acids), vitamins and minerals (Mussatto *et al.*, 2006; McCarthy *et al.*, 2013a). Presently, spent grains are sold as livestock feed, particularly for dairy cattle, with a profit in the range of €1 - €6/ton (Fillaudeau *et al.*, 2006). Other potential applications have been suggested; energy and charcoal production, paper manufacture, use in biotechnological processes and as a human food ingredient (Mussatto *et al.*, 2006).

Meat, eggs, fish, soy and wheat have been highlighted as sources of bioactive peptides (Hartmann & Meisel, 2007; McCarthy *et al.*, 2013b), with protein hydrolysates being utilised in many human nutrition products including energy drinks, weight control and sports nutrition products (Clemente, 2000). In addition to antioxidant and anti-inflammatory potential, protein hydrolysates also possess hypocholesterolemic and anti-hypertensive properties (Hartmann & Meisel, 2007).

Phenolic-rich extracts from BSG have previously demonstrated bioactivity, by protecting against oxidant-induced DNA damage (McCarthy *et al.*, 2012). We previously reported BSG protein isolate and associated hydrolysates possess selective immunomodulatory potential, significantly reducing concanavalin-A (conA) stimulated interferon-gamma (IFN- γ) production (McCarthy *et al.*, 2013c). As a continuation of this research, additional protein hydrolysates were prepared from BSG, with an aim of producing a hydrolysate with enhanced bioactivity. Since molecular weight is one of the main factors influencing biological properties (Jeon *et al.*, 1999; Park *et al.*, 2001), hydrolysates (produced using Alcalase 2.4L, Corolase PP and Flavourzyme) were fractionated on the basis of differences in molecular mass for bioactivity analysis. It has been reported that membrane separation of protein hydrolysates can result in peptide fractions with enriched bioactivity (Korhonen & Pihlanto, 2006). Cellular antioxidant activity assays, representing the first line of antioxidant defence in the body, are essential to identify the ability of compounds to intercept oxidative stress and prevent or reduce oxidation (Sies, 1997). Furthermore, the comet (single cell gel electrophoresis) assay has been shown to be a successful means of determining the chemoprotective effects of novel functional food ingredients, through antioxidant mechanisms, in cell culture (McCarthy *et al.*, 2012; Phelan *et al.*, 2009; Gleis *et al.*, 2006). It is hypothesised that fractionation of BSG protein hydrolysates will produce peptide fractions with enriched bioactivity, measured using common assays of bioactivity in cell culture.

The aims of the present study were to assess: firstly, the cytotoxic effects of a second set of BSG protein hydrolysates in human monocytic blood cells (U937) and human leukaemic T cells (Jurkat T cells); secondly, the cellular antioxidant potential of the BSG hydrolysates in the U937 cell line; thirdly, the ability of BSG hydrolysates to protect against H₂O₂-induced DNA damage in U937 cells; and fourthly, the immunomodulatory potential of BSG hydrolysates in conA stimulated Jurkat T cells.

6.3. Materials and Methods

6.3.1. Materials

All chemicals were purchased from Sigma-Aldrich Ireland Limited, Co. Wicklow, Ireland, unless otherwise stated. U937 and Jurkat T cells were obtained from the European Collection of Animal Cell Cultures (ECACC).

6.3.2. Generation of protein rich isolate and protein hydrolysates

The BSG protein rich isolate was obtained by a method previously described (Connolly *et al.*, 2013). Samples B - T were generated using a BSG protein rich isolate extracted at bench scale whereas samples U - Y were obtained using BSG protein rich isolate extracted at semi-pilot scale. The freeze dried protein rich isolates were then hydrolysed by a range of commercially available enzymes under conditions described in Table 1. The pH was maintained by addition of 0.5 or 2 N 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 hydrolysates were each adjusted to pH 7.0, heated at 95 °C for 10 min to inactivate the enzyme and were then freeze dried or fractionated using 5 and 3 kDa molecular weight cut-off membranes (Minimate™ Tangential Flow Filtration

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

Sample	Description	Enzyme/water	E/S ratio (w/w or v/w) %
B	Control for K-Q	Water	-
C	Control for R-T	Water	-
K	BSG protein hydrolysate	Protamex	1
L	BSG protein hydrolysate	Prolyve	1
M	BSG protein hydrolysate	Protex 6L	1
N	BSG protein hydrolysate	Promod 144MG	1
O	BSG protein hydrolysate	Promod 24P	1
P	BSG protein hydrolysate	Trypsin 250	1
Q	BSG protein hydrolysate	Corolase L10	1
R	BSG protein hydrolysate	Alcalase 2.4L	2.5
S	5 kDa retentate	Alcalase 2.4L	2.5
T	5 kDa permeate	Alcalase 2.4L	2.5
U	BSG protein hydrolysate	Alcalase 2.4L	1
U < 3	3 kDa permeate	Alcalase 2.4L	1
U < 5	5 kDa permeate	Alcalase 2.4L	1
U > 5	5 kDa retentate	Alcalase 2.4L	1
V	BSG protein hydrolysate	Corolase PP	1
V < 3	3 kDa permeate	Corolase PP	1
V < 5	5 kDa permeate	Corolase PP	1
V > 5	5 kDa retentate	Corolase PP	1
W	BSG protein hydrolysate	Flavourzyme	1
W < 3	3 kDa permeate	Flavourzyme	1
W < 5	5 kDa permeate	Flavourzyme	1
W > 5	5 kDa retentate	Flavourzyme	1
X	Unhydrolysed BSG protein	-	-
Y	Control for U-W	Water	-

Capsules, Pall Corporation, New York, USA) and then freeze dried. All samples were prepared at 50 °C (except R, S and T, 60 °C) and pH 7 (except C, R, S and T, pH 9).

6.3.3. Cell culture

A human monocytic blood cell line (U937 cells) and human leukaemic T cell line (Jurkat T cells) were maintained in antibiotic-free RPMI-1640 medium supplemented with 10 % (v/v) foetal bovine serum (FBS), in a 5 % CO₂ atmosphere at 37 °C. Cells were adjusted to a density of 1 x 10⁵ cells/mL for cell proliferation, superoxide dismutase and comet assays and 2 x 10⁵ cells/mL for enzyme-linked immunosorbent assay. Reduced serum media (2.5 % FBS) was used for all experiments.

6.3.4. Cell proliferation

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure cell proliferation. U937 and Jurkat T cells, at a density of 1 x 10⁵ cells/mL, were incubated for 24 hr at 37 °C with protein samples at concentrations of 0 - 10 % (v/v) or 0 - 5 % (v/v), respectively, in a 96-well plate. Colour blanks (sample and media only, without cells) were used to allow for possible colour interference by samples. The MTT assay kit (Roche Diagnostics Limited, West Sussex, England) was used to quantify cell proliferation, based on the concept that metabolically active cells convert the yellow tetrazolium MTT salt to a purple formazan derivative. In brief, 10 µL MTT reagent 1 and 100 µL RPMI media were added to each well and incubated for 4 hr at 37 °C. Subsequently, 100 µL was then removed and 100 µL MTT reagent 2 added, followed by an overnight incubation at 37 °C, before absorbance at 570 nm was measured spectrophotometrically using a microplate reader (Spectrafluorplus, Tecan). GraphPad Prism 4 was used to calculate the concentration of the samples needed to induce 50 %

cell death (IC₅₀ value), expressed as % (v/v). A non-toxic concentration of 0.5 % (v/v) was chosen for further analyses.

6.3.5. Superoxide dismutase (SOD) assay

Using a Calbiochem SOD Assay Kit (Merck Chemicals Ltd., Nottingham, U.K.), SOD activity was measured. U937 cells (5 mL, 1 x 10⁵ cells/mL) were incubated for 24 hr with 0.5 % (v/v) test samples at 37 °C, followed by a 60 min exposure to 100 µM H₂O₂ at 37 °C. SOD activity is expressed relative to the protein content of the samples, measured using the Bicinchoninic Acid Protein (BCA) assay, previously described (Smith *et al.*, 1985), as SOD units/mg protein cell homogenate (U mg⁻¹ protein). The amount of SOD enzyme needed to dismutate 50 % of the superoxide radical (O₂^{•-}) is equivalent to 1 SOD unit.

6.3.6. Single cell gel electrophoresis (SCGE) - Comet assay

U937 cells (2 mL, 1 x 10⁵ cells/mL) were incubated for 24 hr at 37 °C with 0.5 % (v/v) test samples and subsequently exposed to 50 µM H₂O₂ for 30 min. Using the fluorescein diacetate ethidium bromide (FDA/EtBr) assay, cell viability was checked and was consistently > 90 %. The comet assay, as previously described (McCarthy *et al.*, 2012), was used to determine the effect of the extracts on oxidant-induced DNA damage. Briefly, cells were harvested and embedded on microscope slides (prepared with normal gelling agarose (NGA)) using low melting point agarose (LMP). Cells were placed in lysis solution for 1 hr at 4 °C, followed by electrophoresis at 300 mA, 21V for 25 min. Slides were neutralised by using Tris (0.4 M) and stained with EtBr (20 µg/mL). Cells were visualised using a fluorescence microscope (Optiphot-2, Nikon) and Comet 5.5 image analysis software. For each slide, 50 cells were scored and DNA damage expressed as percentage tail DNA.

6.3.7. Enzyme-linked immunosorbent assay (ELISA) – Interferon- γ production

Jurkat T cells were treated with concanavalin-A (conA, 50 μ g/mL) and incubated for 24 hr at 37°C with 0.5 % (v/v) test samples. eBioscience ELISA kit (Human IFN- γ ELISA Ready-Set-Go) was used to measure the production of interferon- γ (IFN- γ). Absorbance was read at 450 nm using a microplate reader (ThermoScientific Varioskan Flash).

6.3.8. Statistical Analysis

All data points represent the mean \pm standard error (SE) of at least 3 independent experiments. Statistical analysis was carried out using GraphPad Prism 4.00 (GraphPad Software, California, U.S.A.); analysis was by one-way analysis of variance (ANOVA) followed by Dunnett's test (or Tukey's multiple comparison test, where stated).

6.4. Results

For hydrolysate samples K-Q and R-T the control protein samples are labelled B and C, respectively, as detailed in Table 1. These control samples, B and C, were previously studied in our earlier publication (McCarthy *et al.*, 2013c). The control for samples U, V, W and associated fractionated hydrolysates is sample Y.

6.4.1. Cell proliferation

The effect of test samples on cell proliferation in both U937 and Jurkat T cells was measured using the MTT assay. Concentrations between 0 - 10 % (v/v) were analysed in the U937 cell line, as shown in Table 2. Sample Y was the most cytotoxic, with 0.5 % (v/v) significantly ($P < 0.05$) decreasing cell proliferation, while M was the least toxic sample in the U937 cell line. The concentration of each sample needed to induce 50 % cell death (IC_{50} value) was calculated. In the U937 cell line this was found to range from

Table 2: Effect of BSG protein hydrolysates (0 - 10 % (v/v)) on cell proliferation in the U937 cell line.

Sample ^a	Cell proliferation (% of control) ^b										IC ₅₀ ^c (% (v/v))
	0 %	0.5 %	1 %	2 %	2.5 %	4 %	5 %	6 %	8 %	10 %	
K	100.00	95.73	89.53	95.15	84.48	80.59	95.85	62.87	31.14*	29.32*	6.02
L	100.00	96.78	94.24	111.87	99.52	74.98	133.34	73.18	37.20*	40.89*	6.01
M	100.00	82.93	113.92	104.41	104.80	105.21	114.64	94.67	64.59	64.75	n/d
N	100.00	96.22	88.21	100.04	100.03	125.80	89.08	75.17	49.97	28.51*	8.13
O	100.00	83.97	89.82	89.69	105.13	86.77	107.16	65.09	54.07	31.39*	5.98
P	100.00	93.94	93.63	87.09	112.62	74.10	103.54	88.07	64.07	25.67*	7.84
Q	100.00	87.43	92.08	70.36	103.53	78.17	87.01	73.79*	52.25*	19.75*	9.07
R	100.00	88.23	98.34	81.01	88.22*	30.72*	17.92*	13.84*	55.21	37.16*	2.90
S	100.00	88.42	81.84	68.11	50.58*	20.48*	31.80	29.04*	55.39	74.18	2.01
T	100.00	82.11	58.56*	43.43*	49.05*	63.81*	43.25*	35.99*	50.55*	33.69*	5.84
U	100.00	80.01	88.26	40.77*	29.13*	44.77*	46.09*	30.21*	44.36*	26.86*	1.94
U < 3	100.00	94.42	109.62	99.04	89.10	62.27	88.79	81.67	54.90*	25.29*	9.78
U < 5	100.00	76.21	103.72	95.68	84.25	86.47	83.97	72.53	53.24	30.04*	9.34
U > 5	100.00	62.93	89.92	76.59	50.91	39.97*	22.64*	18.17*	9.12*	-5.14*	4.27
V	100.00	45.00	114.60	82.22	84.36	25.39*	26.63*	23.92*	19.22*	15.88*	3.53
V < 3	100.00	69.94	66.46	77.75	84.42	68.63	63.76	49.07*	34.61*	23.20*	6.33
V < 5	100.00	60.32*	71.44	73.73	83.21	65.12*	71.20	57.27*	38.03*	25.47*	7.19
V > 5	100.00	57.32	94.43	60.37	38.48*	31.00*	45.52*	32.53*	5.63*	12.12*	4.64
W	100.00	79.36	80.68	23.74*	23.58*	17.90*	24.60*	19.20*	14.47*	10.69*	1.57
W < 3	100.00	74.87	78.81	83.82	71.45	61.35	31.48*	13.66*	16.27*	3.76*	4.59
W < 5	100.00	76.07	77.60	57.24*	54.91*	55.93*	43.07*	34.37*	24.54*	8.04*	5.87
W > 5	100.00	66.23	77.95	68.44	20.71*	12.48*	14.66*	-1.22*	16.41*	-2.31*	2.32
X	100.00	68.15	38.82*	35.89*	48.32*	34.51*	29.27*	31.82*	38.03*	24.68*	3.56
Y	100.00	72.63*	58.92*	38.00*	17.74*	17.68*	17.85*	12.62*	25.05*	-8.22*	1.38

^a^b Values represent the mean \pm SE of three independent experiments. ^c Values relate to the concentration of sample added to the cells, expressed as % (v/v) and were computed using GraphPad Prism 4.00. * Denotes statistically significant (P < 0.05) difference in cell proliferation relative to untreated control U937 cells. Statistical analysis by ANOVA followed by Dunnett's test.

1.38 % (v/v) to non-determinable (n/d). Therefore, for further analyses using U937 cells, a non-toxic concentration of 0.5 % (v/v) was chosen.

The maximum concentration tested was 5 % (v/v) for the MTT assay in the Jurkat T cell line, as results in the U937 cell line suggested that concentrations in excess of 5 % (v/v) to be unsuitable for experimentation, having significant ($P < 0.05$) effects on cell proliferation. Results of the MTT assay in the Jurkat T cell line are shown in Table 3. The samples tested were less cytotoxic in Jurkat T cells than in U937 cells, with the IC_{50} values of 10 samples not determinable. The IC_{50} values of the remaining samples were in the range of 1.15 – 13.82 % (v/v). Similar to the effects in U937 cells, sample Y was the most cytotoxic. Therefore, a non-toxic concentration of 0.5 % (v/v) was chosen for immunomodulatory analysis of the samples in the Jurkat T cell line.

Hydrolysates U, V and W and fractionated hydrolysates of U and V had significantly ($P < 0.01$) higher IC_{50} values than that of control sample Y, in both cell lines; indicating that fractionated hydrolysates, particularly with molecular weight (m.w.) < 3 and < 5 kDa, are less cytotoxic than the parent hydrolysate samples.

6.4.2. Antioxidant activity

The antioxidant potential of the samples was measured by their ability to protect against H_2O_2 -induced damage in U937 cells using two assays; the SOD and comet assays. Exposure of U937 cells to 100 μM H_2O_2 for 60 min resulted in a significant ($P < 0.01$) decrease in SOD activity to 57.24 % (Table 4). Samples L, Q and R significantly ($P < 0.01$) protected against this oxidant-induced reduction in SOD activity. A number of the fractionated hydrolysates also provided significant ($P < 0.01$) protection against H_2O_2 -induced decrease in SOD activity; U < 3 kDa, U < 5 kDa, U > 5 kDa, V > 5 kDa, W < 3 kDa, W < 5 kDa, W > 5 kDa. Protection against DNA damage induced by 50 μM H_2O_2 for 30 min was measured using the comet assay. H_2O_2 significantly ($P < 0.01$) increased

Table 3: Effect of BSG protein hydrolysates (0 - 5 % (v/v)) on cell proliferation in the Jurkat T cell line.

Sample ^a	Cell proliferation (% of control) ^b							IC ₅₀ ^c (% v/v)
	0 %	0.5 %	1 %	2 %	2.5 %	4 %	5 %	
K	100.00	78.32	75.55	65.93	78.85	52.44	59.97	n/d
L	100.00	86.38	80.24	75.17	86.51	53.79*	65.38	n/d
M	100.00	78.59	84.88	83.78	89.78	52.37*	76.53	n/d
N	100.00	83.26	87.10	83.96	97.01	65.28	72.88	n/d
O	100.00	94.31	99.21	84.72	95.50	59.75	83.06	n/d
P	100.00	88.38	98.29	81.82	100.43	73.10	67.78	12.56
Q	100.00	89.00	94.09	89.69	109.40	102.77	114.42	n/d
R	100.00	80.58	85.38	73.63	49.78	49.08	66.36	n/d
S	100.00	80.23	79.59	70.80	60.99	17.81*	15.32*	5.70
T	100.00	89.65	85.04	93.08	69.79*	81.69	80.57	6.59
U	100.00	71.03	69.39	47.85*	16.20*	38.46*	35.80*	1.99
U < 3	100.00	58.47*	57.22*	64.17	66.51	71.60	69.85	2.08
U < 5	100.00	79.23	86.17	72.87	73.00	81.38	65.61	11.44
U > 5	100.00	66.13*	67.39	44.67*	18.13*	21.19*	35.40*	2.00
V	100.00	71.63*	74.82*	68.00*	51.79*	29.34*	33.76*	2.48
V < 3	100.00	70.39*	66.20*	62.59*	62.29*	64.55*	51.23*	7.14
V < 5	100.00	75.49	70.92	64.85*	78.06	70.53	75.72	13.82
V > 5	100.00	60.90*	63.56*	9.52*	12.90*	13.92*	12.87*	3.25
W	100.00	63.54	58.09	46.62*	27.75*	27.90*	27.42*	2.02
W < 3	100.00	67.24	59.24*	65.03	60.78*	68.24	64.55	n/d
W < 5	100.00	78.41	67.05	71.12	66.13	57.69	76.92	n/d
W > 5	100.00	55.89	72.16	50.98	39.98	45.22	39.75	n/d
X	100.00	63.36*	63.65*	19.29*	15.56*	48.11*	73.99	4.12
Y	100.00	70.12	68.48	27.16*	28.60*	24.14*	40.79*	1.15

^{a b} Values represent the mean \pm SE of three independent experiments. ^c Values relate to the concentration of sample added to the cells, expressed as % (v/v) and were computed using GraphPad Prism 4.00. * Denotes statistically significant (P < 0.05) difference in cell proliferation relative to untreated control Jurkat T cells. Statistical analysis by ANOVA followed by Dunnett's test.

Table 4: Ability of BSG protein hydrolysates (0.5 % (v/v)) to protect against H₂O₂-induced oxidative stress in the U937 cell line, measured by superoxide dismutase (SOD) activity.

Sample	SOD activity (U mg ⁻¹ protein)	SOD activity (% of control)
Control	0.36 ± 0.01	100 ± 0.00
H ₂ O ₂ control	0.18 ± 0.00*	57.24 ± 0.70*
K	0.28 ± 0.06	91.73 ± 16.82
L	0.57 ± 0.10 [#]	140.47 ± 9.29 [#]
M	0.32 ± 0.03	94.75 ± 8.22
N	0.28 ± 0.02	82.21 ± 13.00
O	0.30 ± 0.04	87.62 ± 10.60
P	0.34 ± 0.10	94.51 ± 15.53
Q	0.35 ± 0.06	109.24 ± 25.03 [#]
R	0.39 ± 0.02	118.93 ± 13.65 [#]
S	0.32 ± 0.06	91.26 ± 10.44
T	0.30 ± 0.03	88.16 ± 10.43
U	0.24 ± 0.12	67.18 ± 5.38
U < 3	0.36 ± 0.20	101.87 ± 4.86 [#]
U < 5	0.40 ± 0.12	111.51 ± 3.31 [#]
U > 5	0.29 ± 0.09	80.53 ± 4.33 [#]
V	0.22 ± 0.05	62.79 ± 3.53
V < 3	0.23 ± 0.20	65.94 ± 7.12
V < 5	0.24 ± 0.09	67.22 ± 4.09
V > 5	0.33 ± 0.10	91.99 ± 2.81 [#]
W	0.25 ± 0.06	70.78 ± 4.24
W < 3	0.44 ± 0.14	124.39 ± 2.89 [#]
W < 5	0.31 ± 0.22	87.72 ± 3.54 [#]
W > 5	0.39 ± 0.04	108.44 ± 1.81 [#]
X	0.21 ± 0.03	58.29 ± 1.95
Y	0.17 ± 0.03	48.24 ± 3.34

Data represents the mean ± SE of four independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * Denotes statistically significant difference in SOD activity between control and H₂O₂ control (P < 0.05). [#] Denotes statistically significant difference in SOD activity between H₂O₂ control and protein hydrolysate (P < 0.01).

the percentage tail DNA to 41.79 % (Figure 1). The fractionated hydrolysate W < 5 kDa significantly ($P < 0.01$) protected against H_2O_2 -induced DNA damage, while other samples did not exert significant antioxidant effects (Figure 1). However, a general trend was observed indicating that the fractionated hydrolysates reduced the % tail DNA to a greater extent than their corresponding unfractionated samples (Figure 1).

6.4.3. Immunomodulatory potential

ELISA was used to measure the ability of the BSG protein samples to protect against the conA stimulated production of IFN- γ in Jurkat T cells; results are shown in Table 5. Sample K showed most anti-inflammatory potential, significantly ($P < 0.05$) reducing IFN- γ production to 53.28 %. Other samples also significantly ($P < 0.05$) reduced IFN- γ levels by a minimum of 12 % (W) and maximum of 28 % (P). Unfractionated hydrolysates U, V and W significantly ($P < 0.001$) reduced IFN- γ production to a greater extent than the corresponding fractionated samples.

6.5. Discussion

BSG is a low-value co-product of the brewing industry, with a composition of 20 % protein and 70 % fibre (Mussatto *et al.*, 2006). Phenolic extracts of BSG have previously demonstrated antioxidant capacity (McCarthy *et al.*, 2012) and BSG protein hydrolysates have been shown to possess selective anti-inflammatory effects (McCarthy *et al.*, 2013c). The bioactivity of further protein hydrolysates, including fractionated hydrolysates, was the focus of the present study.

The MTT cell proliferation assay was used to determine the cytotoxicity of the samples in both the U937 and Jurkat T cell lines and to indicate an appropriate concentration for further analyses. In agreement with previously reported data for BSG hydrolysates, the present set of hydrolysates were more cytotoxic in U937 than in

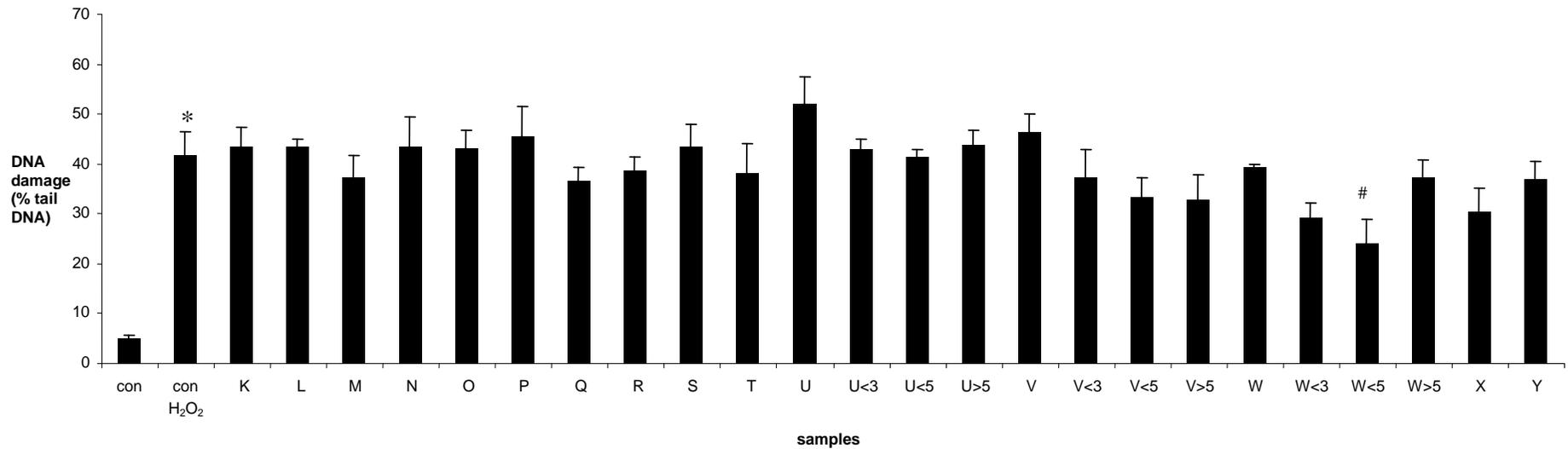


Figure 1: The ability of protein hydrolysates from brewers' spent grain (BSG) to protect against H₂O₂-induced DNA damage in the U937 cell line, measured by the comet assay. Data represents the mean \pm SE of three independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * Denotes statistically significant difference in DNA damage (% tail DNA) between control and H₂O₂ control (P < 0.01). # Denotes statistically significant difference in DNA damage (% tail DNA) between H₂O₂ control and BSG protein hydrolysate (P < 0.01).

Table 5: The effect of BSG protein hydrolysates (0.5 % v/v) on IFN- γ production in concanavalin-A (conA) stimulated Jurkat T cells.

BSG hydrolysate	IFN- γ production (% of control)
	IFN- γ
Control	100.00 \pm 0.00
K	53.28 \pm 17.17*
L	74.28 \pm 4.97
M	76.30 \pm 6.29
N	72.68 \pm 6.74*
O	75.88 \pm 3.84
P	71.16 \pm 8.94*
Q	73.39 \pm 12.66
R	60.67 \pm 13.58
S	58.32 \pm 13.46
T	72.65 \pm 13.44
U	77.70 \pm 2.34*
U < 3	98.19 \pm 2.81 [#]
U < 5	102.56 \pm 3.78 [#]
U > 5	81.95 \pm 2.28*
V	86.86 \pm 1.41*
V < 3	99.55 \pm 2.06 [#]
V < 5	105.57 \pm 3.61 [#]
V > 5	81.32 \pm 2.05*
W	87.27 \pm 1.47*
W < 3	113.47 \pm 4.10 [#]
W < 5	95.15 \pm 3.20
W > 5	78.57 \pm 1.56*
X	83.12 \pm 1.68*
Y	80.88 \pm 4.95*

Data represents the mean \pm s.e. of 3 independent experiments. Statistical analysis by ANOVA followed by Tukey's multiple comparison test. * Denotes statistically significant reduction in IFN- γ production, relative to conA treated Jurkat T cells control (P < 0.05). [#] Denotes statistically significant difference in IFN- γ production, relative to unfractionated parent compound (P < 0.001).

Jurkat T cells (McCarthy *et al.*, 2013c).

Superoxide dismutases (SODs) are a group of three metalloenzymes, copper/zinc (Cu/Zn), manganese (Mn) and iron (Fe) SOD, involved in catalysing the dismutation of the superoxide anion ($O_2^{\cdot -}$) to molecular oxygen (O_2) and H_2O_2 in humans. The assay kit utilised in this study measures all three SODs generated by xanthine oxidase and hypoxanthine. Antioxidant enzymes such as SOD and catalase (CAT) function in minimising oxidative stress. However, the oxidant interception and prevention process is not always entirely effective and products of damage can be continually formed at low levels, leading ultimately to protein and DNA damage, including single-strand breaks (Sies, 1997). The comet assay measures the ability of a sample to protect against oxidant-induced single strand breaks in DNA (DNA damage) *in vitro*. To determine the antioxidant effects of BSG protein hydrolysates, two different antioxidant assays were used – SOD and comet assays, to measure both interception and repair of oxidative stress. A number of protein hydrolysates demonstrated the ability to protect against the H_2O_2 -induced reduction in SOD activity in the U937 cell line (Table 4). Interestingly, the fractionated hydrolysates of U and W had significant antioxidant effects, by the SOD assay ($P < 0.01$), while the parent hydrolysates did not show significant effects. This suggests that the lowest m.w. hydrolysate fractions (< 3 and < 5 kDa) had the greatest antioxidant capability of hydrolysates U - Y. Sample W < 5 kDa was the only BSG protein fraction to show significant ($P < 0.01$) ability to repair DNA, protecting against oxidant-induced DNA damage as assessed by the comet assay. This hydrolysate fraction reduced DNA damage from 41.79 to 21.02 % tail DNA and also increased SOD activity from 57.24 to 87.72 %. The greater antioxidant potential of the lower m.w. (< 5 kDa) hydrolysates supports previous findings in the literature for quinoa seed protein hydrolysates, < 5 kDa (Aluko & Monu, 2003) and rapeseed protein isolates, < 1 kDa (He *et al.*, 2013).

Hydrogen peroxide (H₂O₂) induces oxidative stress in an iron-dependent manner; producing hydroxyl radicals (OH·) via the Fenton reaction. The ability of BSG protein hydrolysates to protect against H₂O₂-induced oxidative stress suggests their ability to scavenge ferrous iron (Fe²⁺), thus making it unavailable for reaction. The ability of proteins to inhibit oxidation by binding iron has been discussed elsewhere (Elisa *et al.*, 2008). In accordance with previously reported data (Kissell & Prentice, 1979), the main amino acids present in the BSG hydrolysates have been found to be in the order of glutamine > proline > leucine (Connolly *et al.*, 2013). Glutamic acid is the amino acid most highly correlated with antioxidant activity of Spanish honeys (Perez *et al.*, 2007). Similarly, pea seed protein hydrolysates with highest antioxidant activity have highest glutamic and aspartic acids contents (Pownall *et al.*, 2010). Hence, the presence of glutamine in the BSG protein hydrolysates may be, at least partially, responsible for their antioxidant activity.

The antioxidant activity of food proteins can be attributable to a number of characteristics. Peptides with 5 – 16 amino acid residues, particularly tyrosine, tryptophan, methionine, lysine, cysteine and histidine are considered to be antioxidant peptides (Sarmadi & Ismail, 2010). Peptide linkage, configuration and concentration, position of amino acids in the peptide sequence, molecular weight and degree of hydrolysis can all contribute to antioxidant activity and the integration of these effects determines overall antioxidant potential (Sarmadi & Ismail, 2010). Furthermore, non-purified food peptides (protein hydrolysates) have higher absorption and antioxidant activity than purified peptides (Sarmadi & Ismail, 2010).

The selective immunomodulatory potential of BSG protein hydrolysates was highlighted in a previously published study (McCarthy *et al.*, 2013c). These hydrolysates demonstrated the ability to reduce the conA stimulated production of IFN- γ in the Jurkat T cells, no effect was seen on interleukins 2 (IL-2), 4 (IL-4) or 10 (IL-10) (McCarthy *et*

al., 2013c). On the basis of these results, it was decided for the purposes of the present study that potential modulation of IFN- γ production would be measured in conA stimulated Jurkat T cells. Hydrolysates K, N, P, U, V and W showed significant ($P < 0.05$) anti-inflammatory activity. Interestingly, hydrolysates U, V and W significantly ($P < 0.01$) reduced IFN- γ production to a greater extent than the low m.w. hydrolysate fractions < 3 and < 5 kDa (except W < 5 kDa). This suggests that unfractionated hydrolysates or fractions with m.w. > 5 kDa possess greatest anti-inflammatory potential. This is further supported by the finding that of the fractionated hydrolysates, only fractions > 5 kDa significantly ($P < 0.05$) reduced IFN- γ production. IFN- γ is a type 2 pro-inflammatory cytokine (Dinarello, 2000) that plays a crucial role in the immune system by secreting chemokines, activating immune cells, regulating antigen presentation and controlling T-lymphocyte adaptive immune responses (McLaren & Ranji, 2009). Hence, the ability to inhibit production of IFN- γ suggests anti-inflammatory activity. Events such as tissue injury, microbial invasion and oxidative stress can induce acute inflammation (Serhan, 2007) mediated by a number of mediators including cytokines and nitric oxide (Feghali & Wright, 1997). A link between antioxidant and anti-inflammatory activities has previously been reported for whey protein hydrolysates (Power *et al.*, 2013; Iskander *et al.*, 2013), and BSG hydrolysates demonstrating anti-inflammatory potential in the present study also significantly increased SOD activity.

In T lymphocytes, it is thought that conA stimulates cytokine production by activating mitogen-activated protein kinase (MAPK) and nuclear factor of activated T cell (NFAT) via a T-cell receptor (Tanaka *et al.*, 2005). Compounds with the ability to suppress cytokine production induced by conA in T lymphocytes can act by inhibiting protein kinase C (PKC) or calcineurin (CN) activity (Tanaka *et al.*, 2005). Hence, BSG protein hydrolysates may be exerting anti-inflammatory effects by these mechanisms.

Chronic inflammation may occur following acute inflammation and result in persistent infection and autoimmune diseases (Serhan, 2007). Such inflammatory and autoimmune diseases include rheumatoid arthritis, inflammatory bowel disease and psoriasis (Simopoulos, 2002). It has been postulated that IFN- γ plays a role in chronic inflammation by acting as a macrophage activating factor (MAF) and a migration inhibition factor (MIF), both activating macrophages and retaining them at the inflammatory site (Feghali & Wright, 1997). Therefore, the discovery of novel anti-inflammatory compounds, particularly with the ability to reduce production of IFN- γ , has implications for human health.

The hydrolysates for the present study were produced by a number of enzymes including Protamex, Alcalase 2.4L, and Flavourzyme (Table 1). Alcalase is a *Bacillus licheniformis* preparation containing subtilisin and glutamyl endopeptidase activities (Spellman *et al.*, 2005). Flavourzyme is from *Aspergillus oryzae* and contains proteinase and exopeptidase activities (Smyth & FitzGerald, 1998). BSG protein hydrolysates M and O prepared with Protex 6L and Promod 24P, respectively, did not show antioxidant or anti-inflammatory potential. Contrastingly, hydrolysates prepared with Alcalase 2.4L, Corolase PP, and Flavourzyme at 50 °C, pH 7 and at an E:S of 1 % (v/v) demonstrated greatest bioactivity. It has previously been reported that there is great variation in the performance of commercial enzymes, with a wide range of protease variants and specificities available (Tavano, 2013; Aspino *et al.*, 2005). Alcalase has been repeatedly documented as being the most effective in terms of degree of hydrolysis (Aspino *et al.*, 2005; Kong *et al.*, 2007; Dong *et al.*, 2008) and bioactivity (Dong *et al.*, 2008; Mao *et al.*, 2011) of resulting hydrolysates. Flavourzyme is also a favourable protease for the production of bioactive protein hydrolysates (Peña-Ramos & Xiong, 2002; Torruco-Uco *et al.*, 2009).

By comparing the results of the current study to those of the previous study of BSG protein hydrolysates (McCarthy *et al.*, 2013c), it is evident that the present set of protein hydrolysates are more bioactive. While the ability to reduce IFN- γ production is demonstrated in the results of both studies, antioxidant activity of BSG protein hydrolysates is only evident in the present study. This may be attributed to differences arising during scaling-up of the initial protein extraction procedure to semi-pilot scale prior to hydrolysis, coupled with the enzymatic conditions (i.e. E/S ratio 1 %, pH 7, temperature of 50 °C) and the fractionation of hydrolysates.

6.6. Conclusion

Results of the present study suggest BSG protein hydrolysates possess antioxidant and anti-inflammatory potential. Hydrolysates with lower molecular weight (< 3 and < 5 kDa) have greatest antioxidant activity, whereas unfractionated hydrolysates or hydrolysates with m.w. > 5 kDa appear to have greater anti-inflammatory effects. These bioactive hydrolysates demonstrate potential for incorporation into functional foods, for the management of oxidation and inflammation associated diseases.

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Chapter 7

A study of the ability of bioactive extracts from brewers' spent grain to enhance the antioxidant and immunomodulatory potential of food formulations following *in vitro* digestion.

7.1. Abstract

The focus of the present study was assessment of the bioactivity of foods fortified with brewers' spent grain (BSG) phenolic extracts or protein hydrolysates, measured following a simulated static gastrointestinal *in vitro* digestion procedure. Snack-bar and chocolate-drink formulations and a commercially available yogurt product were fortified with BSG phenolic extracts (P2 or B2) or BSG protein hydrolysates (W, W < 3 kDa, W < 5 kDa, W > 5 kDa) and subjected to *in vitro* digestion. Cytotoxicity of the fortified food digestates was measured using the (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Non-toxic concentrations of 0.5 % (v/v) and 0.1 % (v/v) digestates were selected for further experiments in Caco-2 and Jurkat T cells, respectively. The comet assay was used to measure the antioxidant effect of fortified food digestates; Caco-2 cells were exposed to 50 μ M hydrogen peroxide (H₂O₂) for 30 min, following incubation for 24 hr with digestates. Yogurt supplemented with B2 digestate significantly (P < 0.05) protected against H₂O₂-induced DNA damage. To measure the immunomodulatory activity of the digestates, Jurkat T cells were stimulated with concanavalin-A (conA) and incubated with digestates for 24 hr prior to determining production of cytokines interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-10 (IL-10) and interferon- γ (IFN- γ) by enzyme-linked immunosorbent assay (ELISA). Bar digestates possessed significant (P < 0.05) immunomodulatory effects. Addition of hydrolysate W significantly (P < 0.05) increased the IFN- γ reducing capacity of the snack-bar. Hydrolysates W < 3 and W < 5 reduced IL-2 production to a greater extent than the unfortified yogurt (P < 0.05). Select BSG extracts possess the ability to enhance the antioxidant and anti-inflammatory potential of food formulations.

7.2. Introduction

Functional foods, which have been described as foods that provide physiological benefits beyond their basic nutrition (Day *et al.*, 2009), are considered to have originated in Japan in the late 1980s (Westrate *et al.*, 2002). Demand by health-conscious consumers has led to the growth of the functional food market and the development of novel functional foods has become a focus of the food industry. Identification of new bioactive compounds increases opportunities for functional food development and provides a greater choice of foods with added health benefits to the consumer. It is imperative that claims regarding functional foods are regulated to guarantee the efficacy and safety of functional foods. In Europe, functional foods are regulated by the Regulation (EC) No. 1924/2006 on nutrition and health claims made on foods (European Commission, 2006, available online). This regulation ensures health claims are supported by scientific data, are non-misleading and pre-approved at the EU level. Additionally, claims should not cause doubt about the safety of other foods or encourage excessive consumption of a particular food.

The addition of bioactive compounds to foods for human consumption allows compounds found in unusual sources or sources without sensory properties to be incorporated into the human diet (Fernandez-Garcia *et al.*, 2009). For a functional food to exert a biological effect *in vivo*, its bioactivity must be retained during digestion. Simulating *in vitro* gastrointestinal digestion is a commonly used technique to mimic gastric and duodenal digestion and to aid in determining the metabolic fate and alterations in bioactivity of potential functional food ingredients during digestion; a crucial step in the development of functional foods.

Brewers' spent grain (BSG) is a nutritionally valuable co-product of the brewing industry, with phenolic extracts and protein hydrolysates from BSG demonstrating antioxidant and anti-inflammatory bioactivities (McCarthy *et al.*, 2012; McCarthy *et al.*,

2013; McCarthy *et al.*, 2013b; McCarthy *et al.*, 2013c). To date, whole BSG has been incorporated into foods such as ready-to-eat snacks and cookies to increase the fibre content (Stojceska *et al.*, 2008; Prentice *et al.*, 1978). However, research regarding the addition of extracts prepared from BSG into foods has not been extensively studied to date. We have previously investigated the effect of the addition of BSG phenolic extracts to fruit juices and smoothies (McCarthy *et al.*, 2013d), however it was found that the initial phenolic content of the beverages was too high for significant increases in antioxidant effects to be evident.

This study aims to incorporate bioactive phenolic extracts and protein hydrolysates from BSG into snack-bar and chocolate-drink formulations and a commercially available yogurt product. After *in vitro* gastrointestinal digestion of the fortified foods, bioactivity of digestates is measured by assessing the antioxidant and immunomodulatory effects using the comet and enzyme-linked immunosorbent (ELISA) assays, respectively.

7.3. Materials and methods

7.3.1. Materials

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich Ireland Limited, Co.Wicklow, Ireland. Caco-2 and Jurkat T cells were obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK).

7.3.2. Preparation of phenolic extracts and protein hydrolysates

Phenolic extracts were prepared from pale and black BSG, as described previously (McCarthy *et al.*, 2012) and designated P1 - P4 and B1 - B4, respectively. For this study, two phenolic extracts were chosen for addition into foods – extract P2 and B2. These

extracts are the supernatants produced following the sequential extraction of BSG using 110 mM NaOH and 1 N NaOH.

The preparation of fractionated protein hydrolysates from BSG has been described previously (McCarthy *et al.*, 2013; McCarthy *et al.*, 2013b). Hydrolysate W, was prepared using 1 % (w/w) Flavourzyme at 50 °C and pH 7.0. The resulting hydrolysate was heated at 95 °C for 10 min to inactivate the enzyme and then freeze dried or fractionated using 5 and 3 kDa molecular weight cut-off membranes (Minimate™ Tangential Flow Filtration Capsules, Pall Corporation, New York, USA) and then freeze dried. Before addition to foods, a 1 % (w/v) hydrolysate solution was prepared using distilled deionised water. Hydrolysates for analysis are labelled W, W < 3 (3 kDa permeate), W < 5 (5kDa permeate) and W > 5 (5 kDa retentate).

7.3.3. Preparation of fortified foods

The snack bar and chocolate drink formulations were obtained from a food company and a yogurt product (Actimel Danone Ltd., Co. Dublin) was purchased in a local supermarket. Following preliminary experiments concentrations of 10, 20 and 30 % (v/w) or (v/v) BSG phenolic extracts were selected for addition to the bar, drink and yogurt, respectively. Protein hydrolysates were added to all foods at a concentration of 2.5 % (v/v).

7.3.4. In vitro digestion

A static gastrointestinal digestion model, as previously described (O'Connell *et al.*, 2007; McCarthy *et al.*, 2013d) was used to simulate human digestion. Briefly, 4 g / 4 mL sample was diluted to a final volume of 8 mL with Hank's balanced salt solution (HBSS). For gastric digestion, pepsin (0.04 g) was added to each sample, followed by a 1 hr incubation in a shaking waterbath (95 rpm) at 37 °C, pH 2. To simulate duodenal

digestion, pancreatin (8 µg) and bile salts consisting of 8 µg glycodeoxycholate, 5 µg taurodeoxycholate and 8 µg taurocholate were added, the pH was adjusted to 7.4 and samples were incubated in a shaking waterbath (95 rpm) at 37 °C for 2 hr. Samples were ultracentrifuged at 53000 rpm for 95 min and the supernatant (digestate) was filtered through a 0.22 µm filter. Before addition to cells, all digestates were sterile filtered (0.22 µm filters), aliquoted into sterile eppendorphs and stored at –80 °C.

7.3.5. Cell culture

Caco-2 cells, human colon adenocarcinoma cells, were maintained in antibiotic-free Dulbecco's Modified Eagle Medium (DMEM), supplemented with 1 % (v/v) non-essential amino acids and 10 % (v/v) fetal bovine serum (FBS), at 37 °C in a 5 % CO₂ atmosphere. Caco-2 cells were seeded at density of 0.5 x 10⁵ and 1 x 10⁵ cells/mL for cell proliferation and comet assays, respectively. Jurkat T cells, a human leukaemic T cell line, were maintained in RPMI-1640 medium supplemented with 10 % (v/v) FBS and plated at a seeding density of 1 x 10⁵ and 2 x 10⁵ cells/mL for cell proliferation and ELISA assays, respectively. Reduced serum media (2.5 % FBS) was used for all experiments.

7.3.6. 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 Caco-2 and Jurkat T cells. Caco-2 cells were allowed to adhere overnight at 37 °C. Cells were incubated for 24 hr at 37 °C with 0 - 50 % or 0 - 5 % (v/v) phenolic or protein fortified food digestates, respectively, in 96 well plates. The MTT assay kit (MTT I proliferation kit, Roche Diagnostics, UK) was then used to measure cell proliferation. MTT reagent 1 (10 µL) was added to cells and incubated for 4 hr at 37 °C. After incubation, 100 µL MTT reagent

2 was added, followed by a final incubation period of 24 hr at 37 °C. Absorbance was determined at 570 nm using a microplate reader (Spectrafluorplus, Tecan) and cell proliferation was calculated as a percentage of the control, untreated cells. On the basis of the MTT assay results, concentrations of 0.5 % (v/v) and 0.1 % (v/v) digestates were selected for further analysis using Caco-2 and Jurkat T cells, respectively.

7.3.7. Comet assay

Caco-2 cells were incubated for 24 hr at 37 °C in a 6 well plate, prior to the addition of digestates (0.5 % (v/v)) and incubation for 24 hr at 37 °C. Following incubation, cells were treated with 50 µM H₂O₂ for 30 min. Cell viability was determined using the fluorescein diacetate ethidium bromide assay (FDA/EtBr). The comet assay was then used to quantify oxidative DNA damage in Caco-2 cells, as previously described (McCarthy *et al.*, 2012; McCarthy *et al.*, 2013; McCarthy *et al.*, 2013b). Briefly, cells were harvested and fixed on microscope slides using low melting point (LMP) agarose, before lysis for 1 hr at 4 °C and electrophoresis for 25 min at 21 V, 300 mA. Slides were neutralised using Tris neutralisation solution, followed by staining with 20 µg/mL ethidium bromide (EtBr) and scoring (50 cells) using Komet 5.5 image analysis software and a fluorescence microscope (Optiphot-2, Nikon). DNA damage was expressed as percentage tail DNA.

7.3.8. Cytokine production

Jurkat T cells were treated with 50 µg/mL Concanavalin-A (conA) and incubated for 24 hr at 37 °C with 0.1 % (v/v) food digestates. Production of interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-10 (IL-10) and interferon-γ (IFN-γ) was measured. Cytokine production was determined using eBioscience ELISA kits (Human Th1/Th2 ELISA Ready-SET-Go kit purchased from Insight Biotechnology, Wembley, UK).

Absorbance was read at 450 nm on a microplate reader (Spectraflourplus, Tecan) and cytokine production expressed as percentage of conA treated Caco-2 cells.

7.3.9. Statistical analysis

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

7.4. Results

7.4.1. Cell proliferation

Caco-2 and Jurkat T cells were incubated for 24 hr with digestates and cell proliferation was measured using the MTT assay (Tables 1, 2, 3 & 4). Non-toxic concentrations of 0.5 % (v/v) and 0.1 % (v/v) digestates were selected for further experiments in Caco-2 and Jurkat T cells, respectively.

7.4.2. Oxidant-induced DNA damage

The ability of the fortified food digestates (0.5 % v/v) to protect against H₂O₂-induced DNA damage in Caco-2 cells was measured using the comet assay. Cell viability was greater than 90 % for all experimental conditions, as determined by the FDA/EtBr assay. Hydrogen peroxide significantly ($P < 0.05$) increased DNA damage to approximately 30 % tail DNA (Figures 1 & 2). The digestate prepared from yogurt supplemented with B2 significantly ($P < 0.05$) protected against DNA damage induced by H₂O₂ and reduced tail DNA to 18.2 % (Figure 1). None of the hydrolysate-fortified foods protected against oxidant-induced DNA damage (Figure 2).

Table 1: Effect of BSG phenolic extract-fortified food digestates on cell proliferation in the Caco-2 cell line.

Sample	Cell proliferation (% of control)							
	0 % (v/v)	2.5 % (v/v)	5 % (v/v)	10 % (v/v)	20 % (v/v)	25 % (v/v)	40 % (v/v)	50 % (v/v)
Digestion blank	100.00 ± 0.00	90.60 ± 7.24	72.49 ± 15.53	68.32 ± 1.24	60.70 ± 15.02	63.93 ± 10.31	21.81 ± 5.47*	0.85 ± 19.19*
Bar	100.00 ± 0.00	17.55 ± 5.26*	10.68 ± 2.77*	0.0 ± 0.0*	1.88 ± 3.83*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
Bar & P2	100.00 ± 0.10	31.05 ± 12.97*	28.26 ± 16.68*	27.88 ± 2.69*	22.88 ± 14.08	21.97 ± 7.08*	17.70 ± 8.95*	0.0 ± 0.0*
Bar & B2	100.00 ± 0.01	21.11 ± 6.12*	7.76 ± 1.98*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
Drink	100.00 ± 0.12	53.98 ± 16.94	61.73 ± 17.43	88.41 ± 26.77	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
Drink & P2	100.00 ± 0.01	50.50 ± 2.43*	30.48 ± 7.36*	3.95 ± 4.41*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
Drink & B2	100.00 ± 0.06	54.10 ± 3.33	56.02 ± 9.82	40.56 ± 4.27	0.33 ± 14.38*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
Yogurt	100.00 ± 0.00	43.38 ± 15.41	51.36 ± 22.56	66.47 ± 22.83	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
Yogurt & P2	100.00 ± 0.01	58.93 ± 12.93	56.43 ± 13.40	37.32 ± 14.86*	33.45 ± 11.08*	44.37 ± 7.63*	20.90 ± 6.74*	0.0 ± 0.0*
Yogurt & B2	100.00 ± 0.01	48.84 ± 10.01*	31.43 ± 6.31*	21.65 ± 3.92*	16.47 ± 5.32*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*

Values are mean ± SE of four independent experiments, expressed as a percentage relative to untreated Caco-2 cells. Statistical analysis by ANOVA followed by Dunnett's Test. * Denotes statistically significant difference in cell viability, relative to untreated Caco-2 cells (P < 0.05).

Table 2: Effect of BSG protein hydrolysate-food digestates on cell proliferation in the Caco-2 cell line.

Sample	Cell proliferation (% of control)					
	0 % (v/v)	0.5 % (v/v)	1 % (v/v)	2 % (v/v)	2.5 % (v/v)	5 % (v/v)
Digestion Blank	100 ± 0.00	96.72 ± 5.45	94.73 ± 15.81	96.82 ± 4.54	106.90 ± 3.69	118.02 ± 4.14
Bar	100 ± 0.00	133.41 ± 12.98	112.16 ± 13.91	65.86 ± 12.79	56.41 ± 11.31	51.01 ± 4.99*
Bar & W	100 ± 0.00	95.71 ± 4.50	80.33 ± 17.76	95.59 ± 0.34	0.0 ± 0.0*	2.98 ± 7.73*
Bar & W <3	100 ± 0.00	109.65 ± 14.21	84.89 ± 1.04	81.63 ± 9.37	49.04 ± 10.94*	45.35 ± 9.05*
Bar & W <5	100 ± 0.00	98.24 ± 5.21	100.52 ± 5.35	96.34 ± 3.78	56.34 ± 11.75	46.84 ± 4.17
Bar & W >5	100 ± 0.00	111.64 ± 17.95	66.06 ± 10.32	24.21 ± 3.85*	28.70 ± 4.11*	34.95 ± 2.89*
Drink	100 ± 0.00	102.50 ± 7.17	84.30 ± 12.12	83.41 ± 19.90	28.89 ± 13.29*	21.98 ± 4.64*
Drink & W	100 ± 0.00	95.36 ± 5.61	94.97 ± 8.19	94.67 ± 5.45	69.58 ± 8.13*	31.16 ± 3.42*
Drink & W < 3	100 ± 0.00	82.35 ± 2.02	85.10 ± 4.95	73.60 ± 11.80	67.43 ± 12.08	67.46 ± 11.13
Drink & W < 5	100 ± 0.00	99.13 ± 4.44	93.71 ± 2.87	73.93 ± 5.61	76.84 ± 6.29*	60.82 ± 3.65*
Drink & W > 5	100 ± 0.00	99.68 ± 4.67	88.94 ± 7.76	91.41 ± 6.03	84.27 ± 2.97	82.89 ± 7.79
Yogurt	100 ± 0.00	137.93 ± 13.10	85.64 ± 9.49	82.90 ± 9.56	52.26 ± 12.41	35.18 ± 9.44*
Yogurt & W	100 ± 0.00	111.96 ± 6.16	102.60 ± 2.56	79.47 ± 0.88	77.78 ± 8.18*	30.81 ± 2.37*
Yogurt & W < 3	100 ± 0.00	94.56 ± 7.53	84.64 ± 7.60	77.71 ± 16.32	71.33 ± 16.09	14.51 ± 8.66*
Yogurt & W < 5	100 ± 0.00	71.98 ± 11.60	79.25 ± 6.65	100.27 ± 3.78	94.91 ± 9.65*	27.78 ± 13.17*
Yogurt & W > 5	100 ± 0.00	98.60 ± 10.13	105.94 ± 6.74	95.63 ± 14.09	89.15 ± 13.68	28.74 ± 8.10

Values are mean ± SE of four independent experiments, expressed as a percentage relative to untreated Caco-2 cells. Statistical analysis by ANOVA followed by Dunnett's Test. * Denotes statistically significant difference in cell viability, relative to untreated Caco-2 cells (P < 0.05).

Table 3: Effect of BSG phenolic extract-fortified food digestates on cell proliferation in the Jurkat T cell line.

Sample	Cell proliferation (% of control)					
	0 % (v/v)	0.5 % (v/v)	1 % (v/v)	2 % (v/v)	2.5 % (v/v)	5 % (v/v)
Digestion blank	100.00 ± 0.00	93.33 ± 2.71	92.33 ± 3.56*	88.95 ± 2.55*	92.71 ± 4.72*	91.33 ± 4.62*
Bar	100.00 ± 0.00	29.96 ± 3.73*	4.16 ± 2.04*	8.90 ± 0.55*	11.82 ± 1.22*	24.92 ± 1.48*
Bar & P2	100.00 ± 0.00	41.94 ± 6.04*	3.28 ± 1.74*	6.32 ± 0.96*	8.94 ± 1.46*	23.07 ± 2.03*
Bar & B2	100.00 ± 0.00	11.91 ± 4.95*	0.0 ± 0.0*	1.73 ± 0.64*	14.64 ± 7.98*	20.15 ± 1.82*
Drink	100.00 ± 0.00	81.12 ± 2.78*	70.66 ± 3.97*	62.19 ± 2.12*	60.94 ± 3.90*	0.0 ± 0.0*
Drink & P2	100.00 ± 0.00	68.00 ± 1.37*	61.75 ± 2.43*	45.23 ± 3.56*	36.53 ± 4.40*	0.0 ± 0.0*
Drink & B2	100.00 ± 0.00	78.11 ± 3.24*	70.43 ± 4.02*	57.75 ± 4.29*	56.35 ± 6.22*	6.76 ± 2.71*
Yogurt	100.00 ± 0.00	58.86 ± 6.14*	36.04 ± 3.96*	0.0 ± 0.0*	0.0 ± 0.0*	3.15 ± 2.88*
Yogurt & P2	100.00 ± 0.00	70.47 ± 3.18*	71.50 ± 3.55*	18.32 ± 9.85*	3.29 ± 0.74*	4.85 ± 1.84*
Yogurt & B2	100.00 ± 0.00	78.31 ± 3.22*	67.43 ± 1.75*	51.99 ± 4.40*	34.69 ± 0.86*	13.49 ± 2.04*

Values are mean ± SE of four independent experiments, expressed as a percentage relative to untreated Jurkat T cells. Statistical analysis by ANOVA followed by Dunnett's Test. * Denotes statistically significant difference in cell viability, relative to untreated Jurkat T cells ($P < 0.05$).

Table 4: Effect of BSG protein hydrolysate-fortified food digestates on cell proliferation in the Jurkat T cell line.

Sample	Cell proliferation (% of control)					
	0 % (v/v)	0.5 % (v/v)	1 % (v/v)	2 % (v/v)	2.5 % (v/v)	5 % (v/v)
Digestion blank	100.00 ± 0.00	97.13 ± 2.75	96.39 ± 4.52	95.50 ± 3.98	97.24 ± 8.38	95.61 ± 8.06
Bar	100.00 ± 0.00	52.70 ± 4.65*	5.55 ± 2.10*	17.68 ± 4.29*	25.39 ± 4.04*	66.28 ± 9.13*
Bar & W	100.00 ± 0.00	12.13 ± 7.58*	9.24 ± 2.63*	32.27 ± 13.53*	28.01 ± 3.81*	50.33 ± 4.94*
Bar & W < 3	100.00 ± 0.00	83.31 ± 4.49*	30.91 ± 2.70*	25.09 ± 1.52*	21.26 ± 1.55*	32.45 ± 1.50*
Bar & W < 5	100.00 ± 0.00	0.0 ± 0.0*	0.0 ± 0.0*	11.48 ± 2.18*	31.59 ± 3.11*	63.46 ± 4.13*
Bar & W > 5	100.00 ± 0.00	81.93 ± 5.11*	37.70 ± 5.33*	28.40 ± 2.95*	19.36 ± 3.37*	18.65 ± 2.72*
Drink	100.00 ± 0.00	73.83 ± 4.26	73.45 ± 8.32	50.67 ± 10.93*	54.53 ± 6.25*	19.62 ± 7.61*
Drink & W	100.00 ± 0.00	110.48 ± 2.64	100.88 ± 5.70	83.36 ± 6.03	58.31 ± 6.25*	0.0 ± 0.0*
Drink & W < 3	100.00 ± 0.00	80.44 ± 0.96	87.75 ± 9.09	88.50 ± 12.91	85.43 ± 12.63	50.24 ± 7.88*
Drink & W < 5	100.00 ± 0.00	102.99 ± 3.62	94.59 ± 7.59	83.93 ± 7.36	77.51 ± 6.97*	0.0 ± 0.0*
Drink & W > 5	100.00 ± 0.00	89.60 ± 4.62	4.81 ± 6.23*	65.13 ± 5.04*	63.64 ± 6.49*	1.65 ± 2.46*
Yogurt	100.00 ± 0.00	79.68 ± 6.65	44.54 ± 6.48	1.26 ± 0.72*	1.33 ± 1.49*	0.17 ± 1.48*
Yogurt & W	100.00 ± 0.00	74.98 ± 4.98*	55.44 ± 9.63*	0.0 ± 0.0*	5.20 ± 2.54*	11.70 ± 4.47*
Yogurt & W < 3	100.00 ± 0.00	88.49 ± 8.06*	92.71 ± 8.28*	34.65 ± 11.84*	0.0 ± 0.0*	0.0 ± 0.0*
Yogurt & W < 5	100.00 ± 0.00	32.37 ± 2.16	0.0 ± 0.0*	0.0 ± 0.0*	4.28 ± 3.31*	3.65 ± 5.06*
Yogurt & W > 5	100.00 ± 0.00	84.67 ± 6.71	76.17 ± 6.08	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Values are mean ± SE of four independent experiments, expressed as a percentage relative to untreated Jurkat T cells. Statistical analysis by ANOVA followed by Dunnett's Test. * Denotes statistically significant difference in cell viability, relative to untreated Jurkat T cells (P < 0.05).

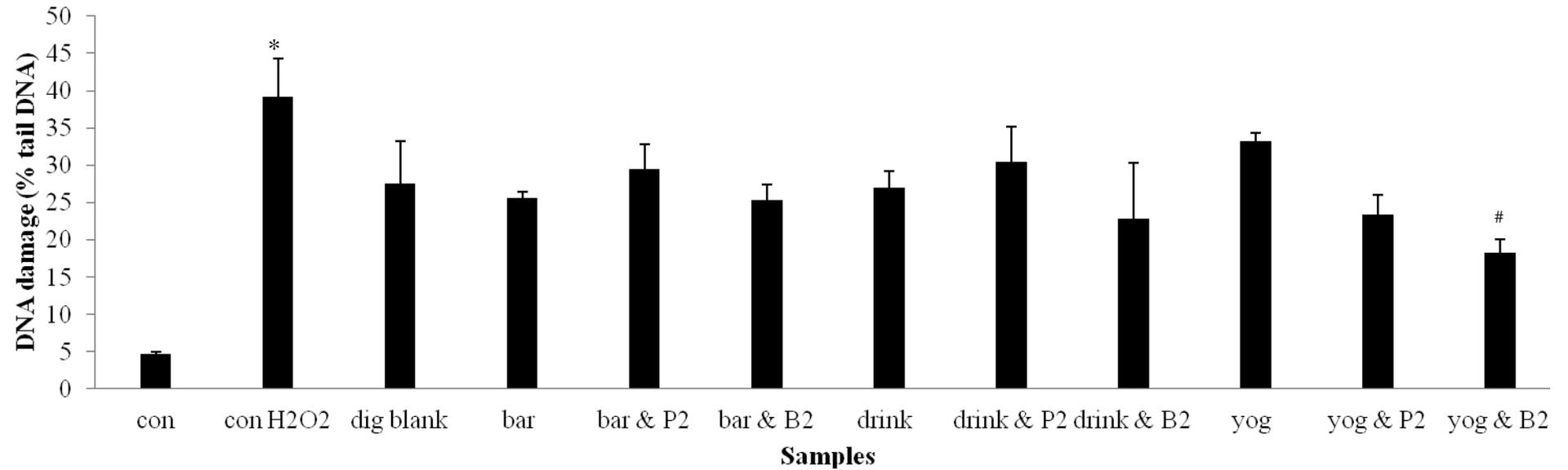


Figure 1: Ability of BSG phenolic extract-fortified food digestates (0.5 % v/v) to protect against H₂O₂-induced DNA damage in the Caco-2 cell line. Data represents the mean \pm SE of four independent experiments. Statistical analysis by ANOVA followed by Dunnett's Test. * Denotes statistically significant difference in DNA damage between control and H₂O₂ control ($P < 0.05$). # Denotes statistically significant difference in DNA damage, relative to H₂O₂ control ($P < 0.05$).

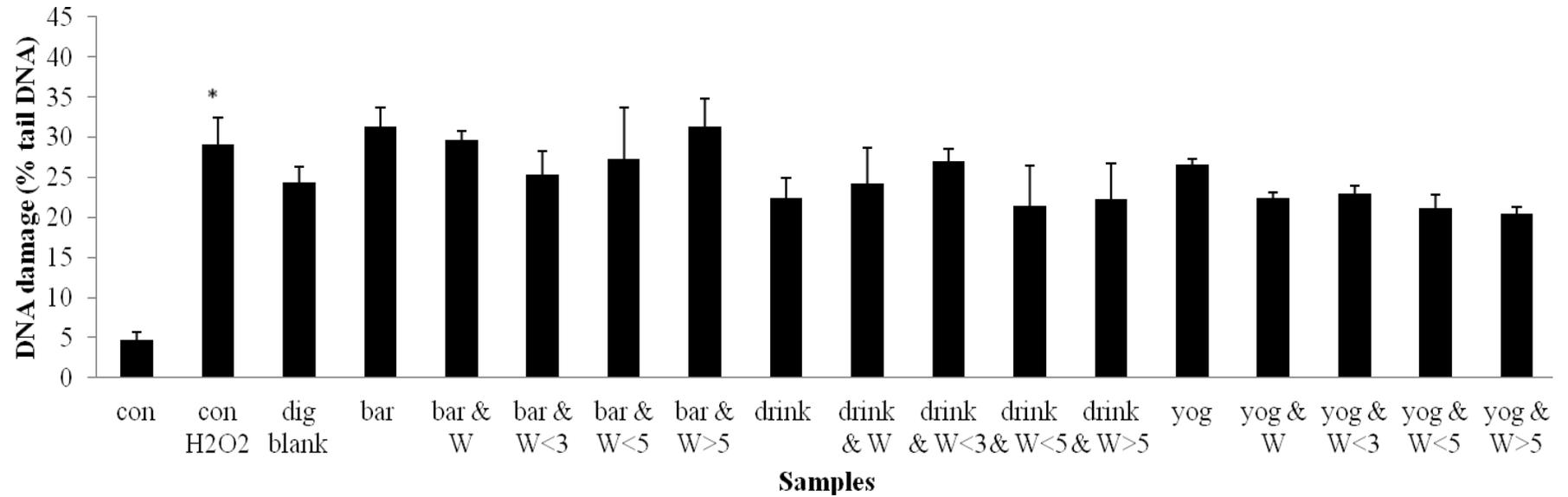


Figure 2: Ability of BSG protein hydrolysate-fortified food digestates (0.5 % v/v) to protect against H₂O₂-induced DNA damage in the Caco-2 cell line. Data represents the mean \pm SE of four independent experiments. Statistical analysis by ANOVA followed by Dunnett's Test. * Denotes statistically significant difference in DNA damage between control and H₂O₂ control (P < 0.05).

7.4.3. Cytokine production

Following stimulation of Jurkat T cells with 50 µg/mL conA in the presence of 0.1 % (v/v) digestates, cytokine production (IL-2, IL-4, IL-10 and IFN-γ) was measured using ELISA. Digestates prepared from the bar, significantly ($P < 0.05$) increased the production of IL-2 and IL-4 but decreased the production of IL-10 and IFN-γ. Similarly, the bar supplemented with P2 increased IL-2 and IL-4 production and decreased IL-10 and IFN-γ production. The bar supplemented with B2 also decreased IFN-γ production but in contrast to the unsupplemented and P2 supplemented bar it decreased the production of IL-2 (Table 5). The drink digestates, both unfortified and fortified, did not alter cytokine production. Unfortified, digested yogurt decreased the production of IL-2 to 66.5 % in Jurkat T cells but this effect was negated by the addition of P2 and B2. There was a significant increase in the production of IL-10 and IFN-γ to 108 % in cells exposed to the digestates of yogurt supplemented with P2 (Table 5).

The snack-bar formulation fortified with protein hydrolysates W, W < 3, W < 5 and W > 5 significantly ($P < 0.001$) reduced IFN-γ production by at least 70 % (Table 6). A number of hydrolysate-fortified digestates also significantly ($P < 0.05$) reduced IL-2 production by as much as 65 %; drink & W > 5, yogurt, yogurt & W < 3, yogurt & W < 5, yogurt & W > 5. IL-4 and IFN-γ production were significantly ($P < 0.05$) increased by bar & W < 5 and drink & W > 5, respectively. Supplementation of the snack bar with hydrolysate W significantly ($P < 0.05$) increased IFN-γ reduction, compared to the unsupplemented snack-bar formulation.

7.5. Discussion

Research regarding the use of BSG in human nutrition has generally been limited to the ability of whole BSG to increase the protein and fibre content of a range of foodstuffs, including cookies, frankfurters and bread (Öztürk *et al.*, 2002; Özvural *et al.*, 2009;

Table 5: Effect of BSG phenolic extract-fortified food digestates (0.1 % v/v) on cytokine production in concanavalin-A (conA) stimulated Jurkat T cells.

Sample	Cytokine Production (% of control)			
	Interleukin-2 (IL-2)	Interleukin-4 (IL- 4)	Interleukin-10 (IL-10)	Interferon- α (IFN- α)
Control	100.00 \pm 0.00	100.00 \pm 0.00	100.00 \pm 0.00	100.00 \pm 0.00
Digestion blank	114.05 \pm 4.81	98.18 \pm 0.35	107.73 \pm 1.01*	101.33 \pm 6.60
Bar	113.17 \pm 7.49*	105.36 \pm 0.36*	88.46 \pm 2.55*	58.09 \pm 4.01*
Bar & P2	114.35 \pm 4.77*	103.52 \pm 0.21*	92.40 \pm 1.58*	64.10 \pm 3.84*
Bar & B2	74.42 \pm 1.94*	101.71 \pm 0.75	95.85 \pm 0.86	63.44 \pm 4.97*
Drink	81.82 \pm 14.55	98.74 \pm 0.34	98.64 \pm 0.24	92.66 \pm 5.51
Drink & P2	103.21 \pm 6.14	98.59 \pm 0.56	99.79 \pm 0.16	114.52 \pm 9.34
Drink & B2	75.11 \pm 3.65	98.20 \pm 0.78	102.42 \pm 0.26	106.37 \pm 9.24
Yogurt	66.15 \pm 10.52*	98.18 \pm 0.52	99.38 \pm 1.66	104.77 \pm 5.42
Yogurt & P2	107.65 \pm 4.23	97.87 \pm 1.47	108.51 \pm 3.19*	108.86 \pm 9.20*
Yogurt & B2	104.93 \pm 6.67	98.63 \pm 0.30	104.62 \pm 1.55	111.63 \pm 6.61

Values are mean \pm SE of three independent experiments, expressed as a percentage relative to Jurkat T cells treated with conA alone. Statistical analysis by ANOVA followed by Dunnett's Test. * Denotes statistically significant difference in cytokine production, relative to control Jurkat T cells ($P < 0.05$).

Table 6: Effect of BSG protein hydrolysate-fortified food digestates (0.1 % v/v) on cytokine production in concanavalin-A (conA) stimulated Jurkat T cells.

Sample	Cytokine Production (% of control)			
	Interleukin-2 (IL-2)	Interleukin-4 (IL- 4)	Interleukin-10 (IL-10)	Interferon- α (IFN- α)
Control	100.00 \pm 0.00	100.00 \pm 0.00	100.00 \pm 0.00	100.00 \pm 0.00
Digestion blank	99.80 \pm 3.86	102.95 \pm 4.60	108.19 \pm 1.25	93.74 \pm 8.85
Bar	95.33 \pm 3.89	130.10 \pm 4.51*	103.22 \pm 2.44	66.10 \pm 7.16
Bar & W	92.68 \pm 7.09	108.13 \pm 5.02	112.29 \pm 9.29	16.83 \pm 10.53* [#]
Bar & W < 3	90.27 \pm 2.01	119.26 \pm 1.82	102.31 \pm 4.90	29.07 \pm 9.46*
Bar & W < 5	95.75 \pm 5.50	127.54 \pm 9.63*	98.48 \pm 2.83	22.23 \pm 11.35*
Bar & W > 5	83.16 \pm 3.26	121.41 \pm 3.17	98.55 \pm 1.77	22.92 \pm 14.65*
Drink	86.37 \pm 1.03	85.17 \pm 12.50	104.62 \pm 1.24	64.97 \pm 11.72
Drink & W	98.00 \pm 7.91	102.86 \pm 1.04	104.30 \pm 0.09	99.75 \pm 7.07
Drink & W < 3	98.77 \pm 5.36	113.04 \pm 9.99	114.30 \pm 10.90	90.15 \pm 10.15
Drink & W < 5	100.79 \pm 5.35	105.95 \pm 1.25	107.20 \pm 1.05	129.01 \pm 10.88
Drink & W > 5	50.29 \pm 2.23*	103.02 \pm 1.01	98.39 \pm 3.24	137.11 \pm 3.94*
Yogurt	75.50 \pm 9.11*	101.79 \pm 1.64	104.02 \pm 3.61	95.62 \pm 9.14
Yogurt & W	102.46 \pm 5.27	98.25 \pm 4056	99.61 \pm 1.93	102.79 \pm 7.67
Yogurt & W < 3	35.91 \pm 9.94* [#]	94.28 \pm 8.10	109.34 \pm 8.75	97.39 \pm 3.94
Yogurt & W < 5	66.44 \pm 1.70* [#]	101.57 \pm 0.66	103.71 \pm 5.40	89.23 \pm 2.31
Yogurt & W >5	76.03 \pm 6.82*	98.23 \pm 2.53	113.72 \pm 3.21	92.00 \pm 0.96

Values are mean \pm SE of three independent experiments, expressed as a percentage relative to Jurkat T cells treated with conA alone. Statistical analysis by ANOVA followed by Dunnett's Test. * Denotes statistically significant difference in cytokine production, relative to control Jurkat T cells ($P < 0.05$). [#] Denotes statistically significant difference in cytokine production, relative to unfortified food ($P < 0.05$).

Stojceska and Ainsworth, 2008). It has previously been shown that phenolic extracts and protein hydrolysates from BSG possess antioxidant and anti-inflammatory effects (McCarthy *et al.*, 2012; McCarthy *et al.*, 2013c). The present research aimed to determine the effectiveness of BSG phenolic extracts and protein hydrolysates as functional ingredients by assessing the bioactivity of extract-fortified foods following an *in vitro* digestion procedure.

The MTT assay was utilised to determine the effect of the food digestates on cell proliferation. In general, fortified food digestates demonstrated higher cytotoxicity in Jurkat T cells, relative to Caco-2 cells. These data demonstrate the varying degrees of sensitivity of different cell lines to cytotoxic compounds. It has been shown that naringenin, a flavonoid, is more cytotoxic in leukocytes, including Jurkat T cells, than in Caco-2 colon cancer cells and the authors suggested this to be in accordance with the fact that anti-cancer drugs are more effective against leukaemia than other cancer types (Kanno *et al.*, 2005). Supporting this, Jurkat T leukaemic cells have repeatedly been reported to be more sensitive to novel copper complex anti-cancer drugs, compared to Caco-2 cells (García-Giménez *et al.*, 2009; Gonzalez-Alvarez *et al.*, 2008).

To evaluate the antioxidant potential of the fortified food digestates, a single cell gel electrophoresis assay was used to measure the ability of the samples to protect against oxidant-induced DNA damage. Hydrogen peroxide (H₂O₂) was used to induce DNA damage, as it is effective in the induction of DNA damage (McCarthy *et al.*, 2012; McCarthy *et al.*, 2013; McCarthy *et al.*, 2013b) by an iron-dependent mechanism (Imlay *et al.*, 1988). Coupling of simulated *in vitro* digestion procedures with bioactivity experiments in intestinal Caco-2 cells, a colon cancer cell line, is a useful model for the investigation of host responses (McCarthy *et al.*, in press), therefore Caco-2 cells were used for measurement of antioxidant activity after *in vitro* digestion of the food samples.

We have previously reported the ability of BSG phenolic extracts P2 and B2 to reduce H₂O₂-induced DNA damage in U937 lymphocytes (McCarthy *et al.*, 2012).

The results of the present study show that the addition of BSG phenolic extract B2 (30 % v/v) to yogurt resulted in a product which significantly ($P < 0.05$) protected against oxidant-induced DNA damage. The unsupplemented yogurt digestate did not demonstrate any antioxidant effects. Similarly, the addition of BSG phenolic extracts increased the antioxidant activity of a juice with a low initial antioxidant activity, as measured by the FRAP assay (McCarthy *et al.*, 2013d). None of the protein hydrolysate fortified foods demonstrated antioxidant effects, although it was previously shown that hydrolysate W < 5 kDa could protect against H₂O₂-induced DNA damage in the U937 cell line (McCarthy *et al.*, 2013b). Antioxidant effects of fortified products may be governed by synergistic and antagonistic reactions between the foodstuff and the BSG extract. Interactions that occur between different ingredients within foods are not fully understood and further investigation is required for the successful development of functional foods.

Treatment of Jurkat T cells with concanavalin-A (conA) stimulates cytokine production in leukocytes, which can be measured by an enzyme-linked immunosorbent assay (ELISA). In the foods supplemented with BSG phenolic extracts (P2 or B2), the bar digestates demonstrated the greatest immunomodulatory effects. Bar & P2, bar & B2 and unfortified bar digestates significantly ($P < 0.05$) reduced IFN- γ production to 58, 64 and 63 %, respectively (Table 5). The snack-bar formulation fortified with protein hydrolysates also demonstrated anti-inflammatory effects, significantly ($P < 0.05$) reducing IFN- γ production by at least 71 % (Table 6). Many of the hydrolysate fortified food digestates also reduced the production of the pro-inflammatory cytokine IL-2; drink & W > 5, yogurt & W < 3, yogurt & W < 5, yogurt & W > 5.

BSG phenolic extract P2 has previously been shown to significantly ($P < 0.05$) reduce production of IL-2, IL-4, IL-10 and IFN- γ , while extract B2 did not demonstrate

anti-inflammatory effects prior to *in vitro* digestion (McCarthy *et al.*, 2013c). Similarly, protein hydrolysates W and $W > 5$ (but not $W < 3$ or $W < 5$) significantly reduced IFN- γ production in Jurkat T cells (McCarthy *et al.*, 2013b). Therefore, the addition of BSG phenolic extracts and protein hydrolysates to foodstuffs, followed by *in vitro* digestion of the foods resulted in greater immunomodulatory effects than was observed for the extracts prior to their addition to a foodstuff.

The addition of bioactive polyphenolic extracts and protein hydrolysates into foods has been extensively researched. Grape seed extracts have been incorporated into full-fat and non-fat yogurts, resulting in products with higher polyphenol content and enhanced anti-radical and antioxidant activities (Chouchouli *et al.*, 2013). Similarly, addition of grape seed extract to bread increases antioxidant capacity in a dose-dependent manner (Peng *et al.*, 2010). Espresso coffee brew supplemented with hazelnut skin extracts possess increased *in vitro* (DPPH radical scavenging) and *in vivo* (ferric reducing antioxidant power (FRAP) of plasma) anti-radical activity (Contini *et al.*, 2012). Synergism between coffee brew phenolics and the added hazelnut skin extracts was suggested (Contini *et al.*, 2012). Whey hydrolysate beverages have shown the ability to decrease low-density lipoprotein (LDL) cholesterol and blood pressure *in vivo* (Fleugel *et al.*, 2010).

This study has shown that the addition of BSG extracts to foods, in particular the snack-bar, can result in a fortified food with selective bioactive potential. Further research to determine the mechanisms involved in the bioactive effects of the fortified foods would facilitate the selection of ingredients and host foods for optimal bioactivity. The fortification of foods with BSG extracts presents a unique opportunity for the utilization of this co-product of the brewing industry; producing a nutritionally-enriched foodstuff, increasing the commercial value of BSG and reducing waste.

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Chapter 8

General Discussion

Functional foods have the ability to provide consumers with a health benefit supplementary to the basic nutrition of the product. There has been considerable efforts to search for bioactive ingredients due to health-conscious consumers demanding healthier versions of foods. Oxidation and inflammation are associated with disease states such as cancer, cardiovascular disease, rheumatoid arthritis and Alzheimer's disease (Valko *et al.*, 2007; Lucas *et al.*, 2006; Chung *et al.*, 2000), hence the ability of antioxidant and anti-inflammatory agents to reduce the risk of these disease states is of significant public health importance.

In recent years, there has been an unprecedented demand for plant-derived bioactive ingredients for incorporation into foods for human nutrition. Brewers' spent grain (BSG), a nutritious co-product of the brewing industry, represents a unique source of novel, potentially bioactive ingredients. Currently, use of BSG is mainly restricted to animal feed, although research on the inclusion of whole BSG into foods such as cookies, bread and frankfurters has been successfully carried out to improve the nutritional profile of these foods (Prentice *et al.*, 1978; Öztürk *et al.*, 2002; Stojceska and Ainsworth, 2008; Özvüral *et al.*, 2009).

The focus of the presented research was to determine the bioactivity of extracts from BSG and the retention of bioactivity in fortified food products. Phenolic extracts and protein hydrolysates were prepared from BSG and subjected to *in vitro* cellular bioactivity assays. Cell culture models used to assess bioactivity are a highly effective, ethical and economical alternative to animal or human trials. However, since complex *in vivo* conditions can not be wholly replicated *in vitro*, human trials are necessary for the substantiation of health claims relating to functional foods. A vast range of cell lines are available and for this research U937 (a human monocytic blood cell line), Jurkat T (leukaemic T lymphocytes) and Caco-2 (colorectal adenocarcinoma cell line) cells were chosen for assay.

Phenolic extracts from black BSG (barley roasted to 200 °C prior to brewing) demonstrated the ability to protect against DNA damage induced by hydrogen peroxide (H₂O₂), measured using the comet assay in U937 cells. By the same assay, phenolic extracts with the highest total phenolic content (TPC; P2, P3, B2, B3) reduced 3-morpholinonydnonimine hydrochloride (SIN-1) induced DNA damage. The failure of the extracts to protect against DNA damage induced by *tert*-butylhydroperoxide (*t*-BOOH) or 4-nitroquinoline oxide (4-NQO), suggests that the demonstrated antioxidant effects could possibly be due to iron chelation, since H₂O₂ and SIN-1 induce oxidative stress by an iron dependent mechanism, unlike *t*-BOOH (Ca²⁺-dependent) and 4-NQO (Cu²⁺-dependent) (Yamamoto *et al.*, 1993; Inoue & Kawanishi, 1995; Kruszewski *et al.*, 2008).

Hydroxycinnamic acids (HA) have previously been shown to be the predominant phenolic compounds present in BSG (Szwajgier *et al.*, 2010). High performance liquid chromatography (HPLC) coupled with diode array detection (DAD) and mass spectrophotometry (MS) was used to determine the phenolic profile of starting barley, whole BSG and BSG phenolic extracts. The HA concentrations were in the order of ferulic acid > *p*-coumaric acid derivatives > ferulic acid derivatives > *p*-coumaric acid > caffeic acid > caffeic acid derivatives. Caffeic acid was the only free phenolic acid (present in aqueous extract P1), while other HA acids were present in the bound form. Comparison of the phenolic profiles of barley versus BSG and pale versus black BSG suggests that both brewing and roasting decreased the HA concentration. In the same study, the 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) scavenging activity and TPC of all samples was measured. While black BSG had a lower HA concentration, a higher DPPH activity and TPC was recorded, suggesting antioxidant activity by a compound other than HA, since the Folin-Ciocalteu method of TPC determination measures any reducing compounds (Huang *et al.*, 2005). The

greater antioxidant activity recorded for black BSG extracts may be attributable to Maillard reaction products (MRPs) formed from non-enzymatic browning during the roasting of barley.

The most bioactive hydrolysates, P2 and B2, were chosen for addition into fruit juices and smoothies and the fortified foods were subjected to *in vitro* digestion, before bioactivity analysis of digestates. Addition of the extracts significantly increased the ferric reducing antioxidant power (FRAP) of cranberry juice, the sample with lowest TPC prior to fortification. There was significantly decreased TPC and antioxidant activity after *in vitro* digestion, however it is important to note that phenolic compounds can exert protective effects in the gastrointestinal tract, before absorption (Halliwell *et al.*, 2000).

Using an enzyme-linked immunosorbent assay (ELISA), phenolic extracts P2 and P3 demonstrated anti-inflammatory potential that may be related to the HA content. P2 and P3 have highest HA concentration, which have previously been reported to have anti-inflammatory effects (Youdim *et al.*, 2002). Six of the studied phenolic extracts (P1 - P3, B2 - B4) were found to possess antioxidant capabilities, measured by cellular antioxidant activity assays – superoxide dismutase (SOD) activity, catalase (CAT) activity and glutathione (GSH) content. While anti-inflammatory potential of the BSG phenolic extracts may be attributable to the HA content, it is speculated that the antioxidant activity is related to the TPC of the extracts.

Protein samples from BSG, including protein isolate (A), protein hydrolysates (D – W) and fractionated protein hydrolysates (U, V and W, < 3, < 5 and > 5 kDa) were screened for their antioxidant and anti-inflammatory effects. Initial analysis of hydrolysates A – J found that BSG hydrolysates did not possess antioxidant activity or inhibit production of the cytokines interleukin 2 (IL-2), 4 (IL-4) or 10 (IL-10). The

ability of the BSG protein samples to selectively reduce production of pro-inflammatory IFN- γ , but not anti-inflammatory IL-4 or IL-10, is an attractive property for nutraceutical development. Fractionated protein hydrolysates demonstrated greatest antioxidant activity with fractionated hydrolysates of U and W providing protection against H₂O₂-induced reduction in SOD activity, while hydrolysate W < 5 kDa reduced H₂O₂-induced DNA damage. Unfractionated U, V and W inhibited concanavalin-A (conA) stimulated interferon- γ (IFN- γ) production to a greater extent than fractionated hydrolysates. It is important to note that the fractionated hydrolysates found to possess anti-inflammatory activity, also demonstrated antioxidant activity by the SOD assay. Oxidative stress has been linked to acute inflammation mediated by cytokines (Serhan, 2007; Feghali and Wright, 1997) and a link between antioxidant and anti-inflammatory capabilities has also been reported for whey protein hydrolysates (Power *et al.*, 2013; Iskander *et al.*, 2013).

On the basis of cellular bioactivity assay results, bioactive phenolic extracts (P2 and B2) and protein hydrolysates (W, W < 3 kDa, W < 5 kDa, W > 5 kDa) were chosen for incorporation into snack-bar and chocolate-drink formulations and a commercially available yogurt product. Black BSG extract B2 increased the antioxidant capacity of the formulation with lowest initial antioxidant activity; a result similar to that reported for inclusion of BSG extracts into fruit beverages (Chapter 3). Snack-bar digestates showed greatest immunomodulatory potential and addition of BSG protein hydrolysate W significantly (P < 0.05) enhanced the IFN- γ reducing capability of the snack-bar formulation. Interestingly, addition of hydrolysates W < 3 or W < 5 to yogurt, followed by *in vitro* digestion, significantly (P < 0.05) increased the IL-2 reducing ability of the yogurt, despite W < 3 and W < 5 demonstrating no anti-inflammatory effects prior to the fortification study (Chapter 6). This suggests

either a synergistic effect between the yogurt and $W < 3$ and $W < 5$ or an increase in bioactivity following *in vitro* digestion.

This evidence contributes to existing research regarding the use of BSG as a functional food ingredient. To date, research on BSG for human nutrition has been focused on whole BSG and this novel data on the *in vitro* bioactivity of phenolic extracts and protein hydrolysates progresses this area of research.

The present thesis has shown the bioactive potential of BSG phenolic extracts and protein hydrolysates in cell culture model systems. This *in vitro* approach has both strengths and limitations. Cell culture model systems are economical, ethical, highly effective and allow a high throughput and reproducibility. A vast selection of cell lines are available for experimentation, conditions are well defined and *in vitro* studies give reliable data on initial efficacy and mechanisms of action. Limitations of *in vitro* studies include the use of homogenous cell lines (heterogenous *in vivo*), lack of circulating factors including hormones and signalling molecules and the use of concentrations in excess of levels in systemic circulation. *In vivo* studies are considered the “gold standard” and are essential for accurately determining bioactive effects in the body, using concentrations of biological relevance and accounting for bioavailability and metabolism.

The incorporation of these preparations into formulated foods shows selective bioactivity retention following *in vitro* digestion. These novel preparations possess characteristics that favours their incorporation into functional foods for human consumption. Since phenolic extracts and protein hydrolysates demonstrated varying degrees of antioxidant and anti-inflammatory potential, future research should focus on the formulation of a combined phenolic and protein ingredient blend for human ingestion. A further 3 years of research funding has been granted to continue the research in this area. This work will optimise the extraction of phenolic extracts and

protein hydrolysates and determine their bioactivity *in vitro*. The technofunctional, bitterness, physicochemical and proteomic characteristics of ingredients will be determined and beverages containing phenolic and protein extracts will be formulated and flavoured for a human intervention trial and biomarkers of antioxidant and anti-inflammatory activity assessed.

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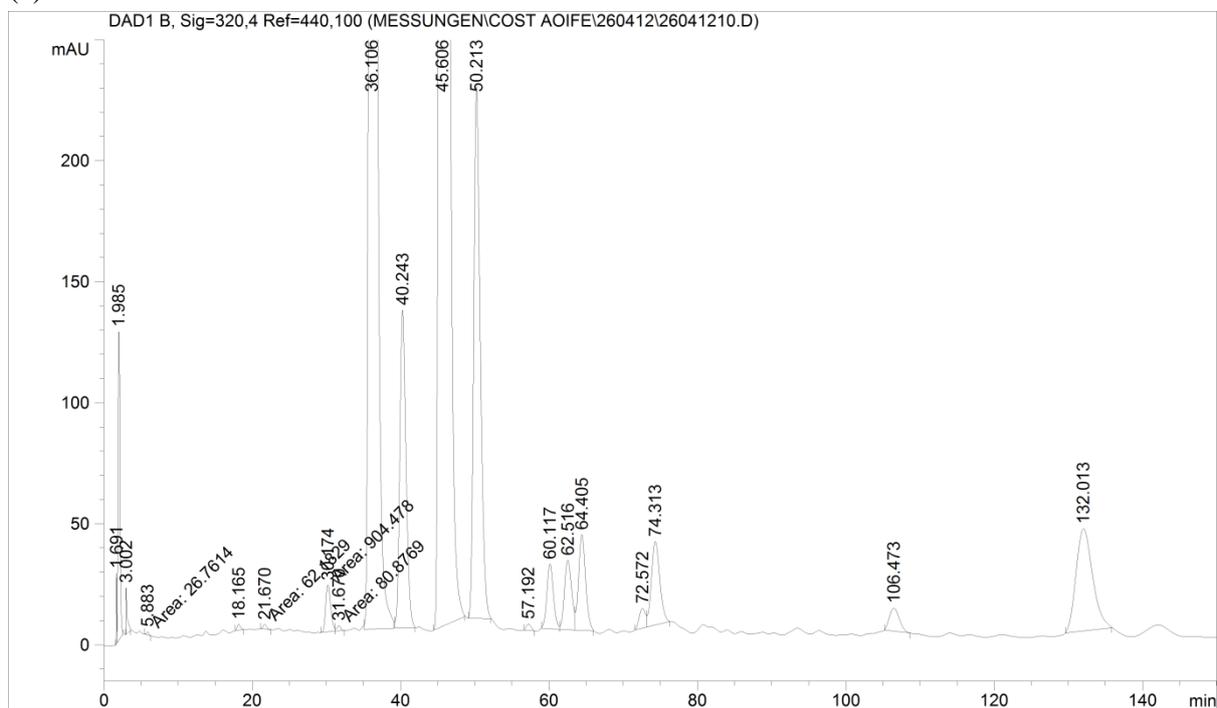
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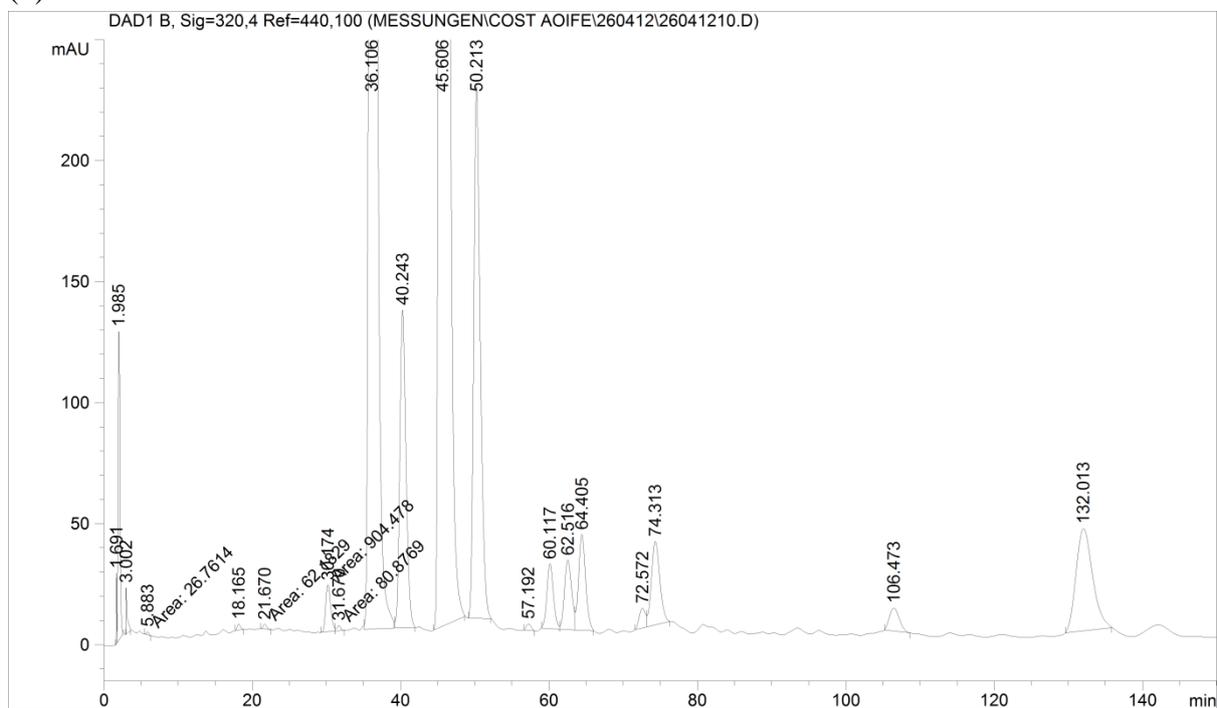
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Appendices

(c)



(d)



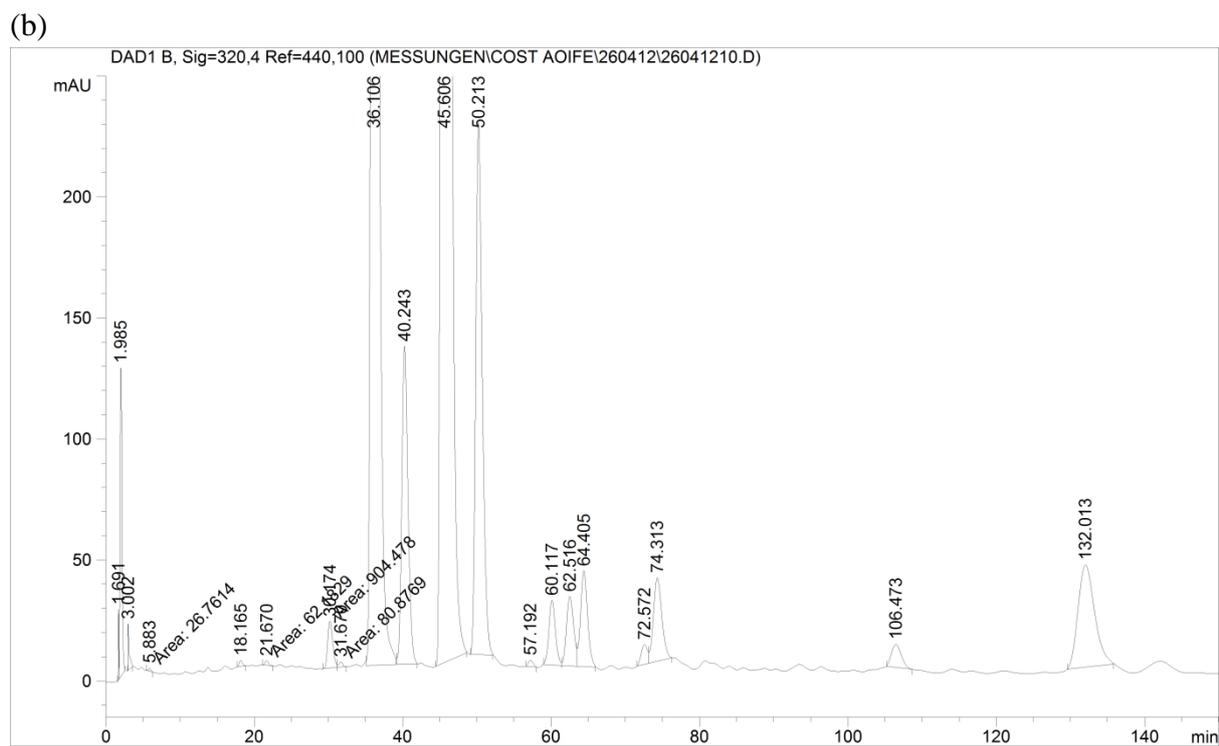
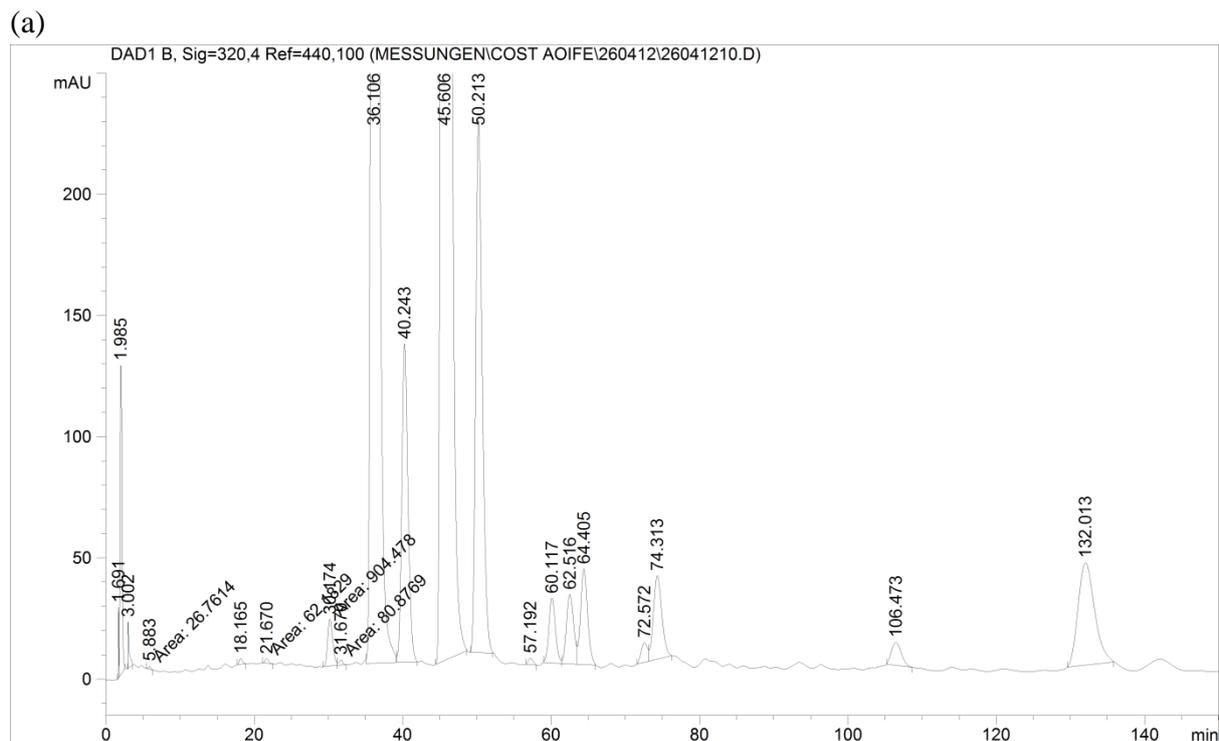
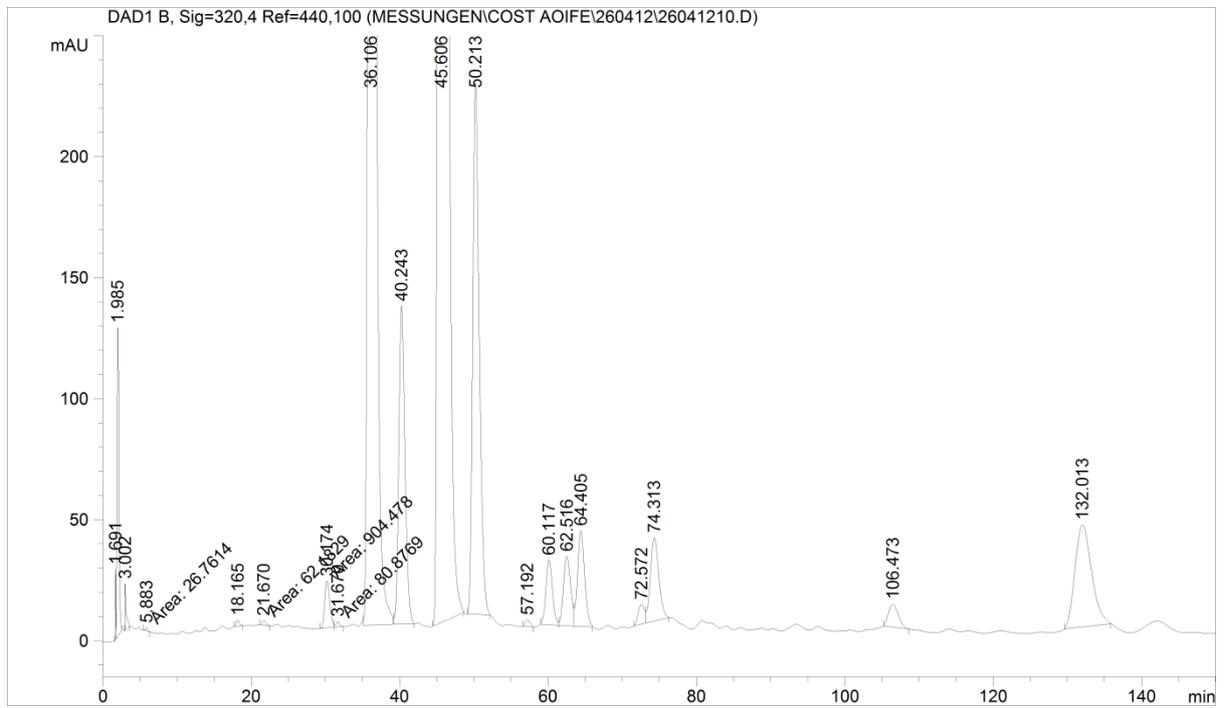


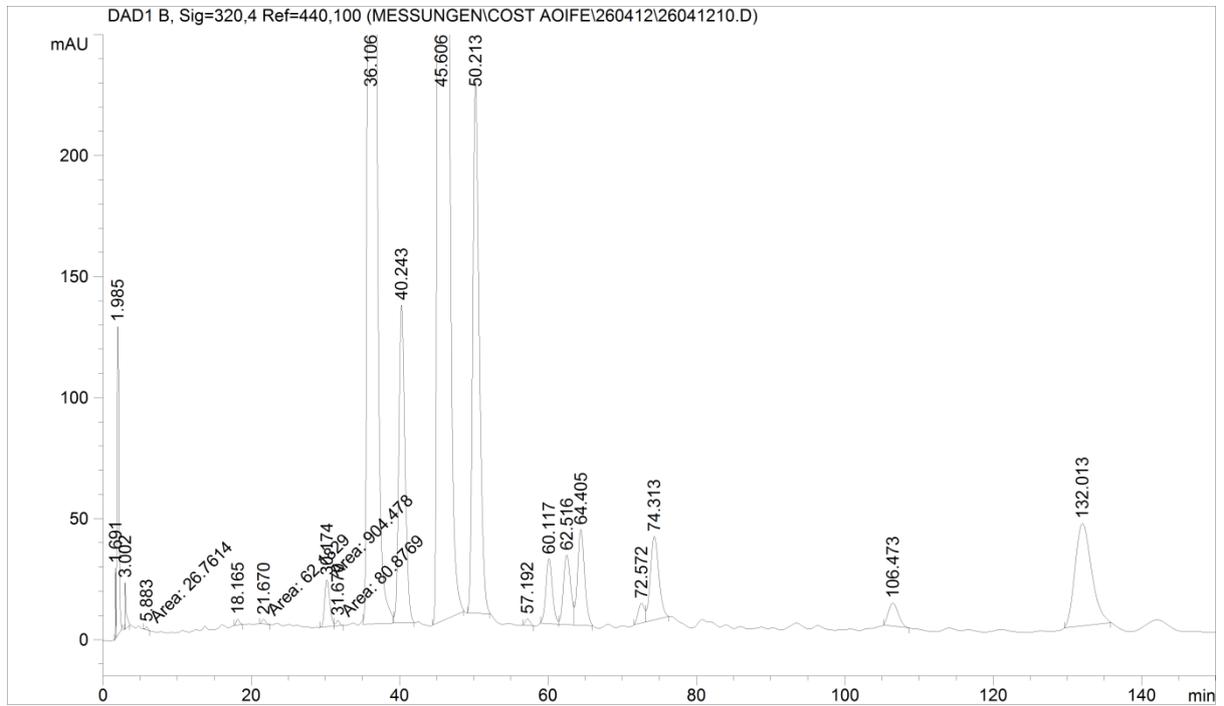
Figure 2: HPLC chromatogram of phenolic extracts from black brewers' spent grain (BSG);

(a) B1 (b) B2 (c) B3 (d) B4. Conditions of HPLC analysis are described in Chapter 3.

(c)



(d)



Conference on 'Translating nutrition: integrating research, practice and policy' Postgraduate Symposium

Brewers' spent grain; bioactivity of phenolic component, its role in animal nutrition and potential for incorporation in functional foods: a review

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Brewers' spent grain (BSG) is a low-value co-product of the brewing industry produced in large quantities annually. This paper reviews the existing evidence regarding the phenolic component of BSG, focusing on composition, extraction and biofunctions such as antioxidant, anti-atherogenic, anti-inflammatory and anti-carcinogenic activities. Furthermore, the incorporation of BSG in foodstuffs will be discussed, including the use of BSG as an animal feed supplement and the potential of BSG to be incorporated into foods for human consumption. BSG contains hydroxycinnamic acids including ferulic acid, *p*-coumaric acid and caffeic acid; which have shown bioactivity in the pure form (antioxidant, anti-inflammatory, anti-atherogenic and anti-cancer). Phenolic extracts from BSG have also shown antioxidant potential, by protecting against oxidant-induced DNA damage, possibly by Fe chelation. Studies show that BSG has many benefits when used as a supplement in animal feed, such as increasing milk yield, milkfat content and in providing essential dietary amino acids. The incorporation of BSG in human foods such as cookies and ready-to-eat snacks has resulted in increased protein and fibre contents of the products, where the changes in organoleptic properties are controllable. It can be concluded that the phenolic component of BSG has potential bioactive effects, which are worth pursuing given that the inclusion of BSG into human foodstuffs is viable and beneficial.

Brewers' spent grain: Phenolic acids: Animal nutrition: Functional foods: Bioactivity

Brewers' spent grain (BSG) is the solid fraction of barley malt remaining after the production of wort. According to the Eurostat Data, BSG is the main by-product of the brewing industry, with approximately 3.4 million tonnes being produced annually in the EU⁽¹⁾, at least 160 000 tonnes of which are produced in Ireland.

This solid residue contains water insoluble proteins in addition to the husk, pericarp and seed coat of the original barley grain⁽²⁾. Protein and fibre account for 20 and 70% of BSG dry matter, respectively, while the starch content of BSG is insignificant (due to the absence of starchy endosperm). Owing to its protein-rich composition, BSG

has the potential to be utilised in a manner similar to whey protein, providing health benefits for consumers. BSG is also rich in phenolic compounds, particularly ferulic acid and *p*-coumaric acid⁽³⁾, along with oligosaccharides and polysaccharides⁽⁴⁾. Emerging evidence, with regard to the ability of dietary phenolic compounds to exhibit anti-carcinogenic, anti-inflammatory and antioxidant activities^(5,6) has led to significant interest in plant phenolic compounds particularly by the food industry, scientists and consumers.

To date, BSG has been widely used as an animal feed, particularly for cattle, to provide high amounts

Abbreviations: BSG, brewers' spent grain; COX-2, cyclooxygenase-isoform 2; DPPH, 1,1-diphenyl-2-picrylhydrazyl.

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Table 1. The approximate chemical composition of brewers' spent grain

Study	Component (as % dry matter*)						
	Protein	Lipid/Oil	Ash	Cellulose	Hemicellulose	Lignin	Starch
Beldman <i>et al.</i> ⁽⁹⁾	23.8	–	3.5	15.1	24.8	–	2.0
Valverde ⁽¹⁰⁾	24.0	6.0	–	17.0	39.0	4.0	–
Kanauchi <i>et al.</i> ⁽¹¹⁾	24.0	10.6	2.4	25.4	21.8	11.9	–
Santos <i>et al.</i> ⁽¹²⁾	24.2	3.9	3.4	–	–	–	–
El-Shafey ⁽¹³⁾	26.7	8.9	3.9	–	–	5.3	–
Mussatto & Roberto ⁽¹⁴⁾	15.3	–	4.6	16.8	28.4	27.8	–
Xiros ⁽¹⁵⁾	14.2	13.3	3.3	12.0	40.2	11.5	–
Treimo ⁽¹⁶⁾	23.4	–	–	–	–	12.6	7.8

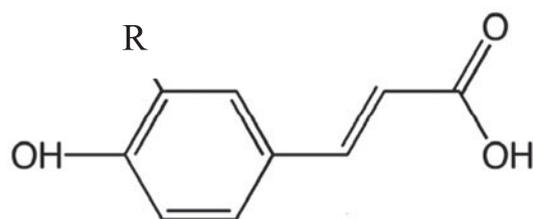
*Values expressed as % dry matter, which has been documented in two studies to be 20.4⁽⁹⁾ and 20%⁽¹³⁾.

of both protein and fibre. BSG is an excellent feed ingredient for ruminants, providing all the essential amino acids when combined with inexpensive N sources such as urea⁽⁷⁾. However, with the increased cost of disposal of the solid fraction, alternative uses are highly sought-after and it has been shown that BSG can be effectively integrated into ready-to-eat snacks to increase dietary fibre, crude protein and fat levels⁽¹⁾. Other areas of successful research include the blending of BSG with flour for incorporation into cookies⁽⁸⁾ and the addition of BSG to dough to improve the dietary fibre content in bread⁽¹⁾.

This review details the existing evidence regarding BSG. A specific focus is placed on the potential bioactivities of phenolic compounds (particularly ferulic acid and *p*-coumaric acid) present in BSG, and the incorporation of BSG into foodstuffs, for both human and animal consumption.

Composition of brewers' spent grain

Many studies have reported on the approximate composition of BSG, which contains protein, fat, cellulose, hemicellulose and lignin (Table 1). As shown, there is good consistency with regard to the composition of BSG. However, variations can arise due to differences in barley variety, harvesting time, characteristics of hops added and brewery technology⁽¹²⁾. BSG predominantly consists of the husk-pericarp-seed coat layers that are rich in cellulose, non-cellulosic polysaccharides, lignin, protein and fat. This is reflected in the composition of BSG (Table 1), and thus BSG can be regarded as a lignocellulosic material⁽⁴⁾. In addition to the components detailed in Table 1, it has been shown that BSG is also a valuable source of vitamins, minerals and amino acids, particularly for animal feeding. The vitamins present in BSG are biotin, folic acid, niacin, choline, riboflavin and thiamine, pantothenic acid and pyridoxine⁽⁷⁾. BSG is also reported to contain minerals such as Ca, Cu, Fe, Mn, K and Na^(7,17) and both essential (including lysine, histidine, methionine, phenylalanine, tryptophan) and non-essential (including alanine, serine, glycine, proline) amino acids⁽⁷⁾. When combined with inexpensive N sources, such as urea, BSG can provide all the essential amino acids to ruminant animals⁽⁷⁾.



ferulic acid (C₁₀H₁₀O₄)

R = OCH₃

p-coumaric acid (C₉H₈O₃)

R = H

Fig. 1. The general structure of hydroxycinnamic acid and the functional groups for ferulic and *p*-coumaric acids.

Phenolic component of brewers' spent grain

Phenolics present in brewers' spent grain

Phenolic acids, particularly hydroxycinnamic acids and hydroxybenzoic acids are secondary plant metabolites found extensively in plant foods. Phenolic acids are currently the focus of much attention due to their potential to act as antioxidant, anti-inflammatory and anti-carcinogenic compounds^(5,6).

As previously mentioned, BSG consists predominantly of the husk-pericarp-seed coat and is largely made up of cell walls. Since most of the phenolic compounds of the barley grain are contained in the husk⁽⁴⁾ and hydroxycinnamic acids accumulate in the cell walls, BSG is a potentially valuable source of phenolic acids.

There is evidence to suggest that ferulic acid and *p*-coumaric acid (as shown in Fig. 1) are present at relatively high concentrations in BSG⁽³⁾. Some of the existing literature regarding the presence of ferulic and *p*-coumaric acid is detailed in Table 2. Ferulic acid was found to be the most abundant hydroxycinnamic acid being present at concentrations ranging from 1860 to 1948 µg/g, while the *p*-coumaric levels ranged from 565 to 794 µg/g⁽²³⁾. More recent evidence shows that BSG consists of 1.16% mono and dimeric phenolic acids, with 53% of the monomeric phenolic acids accounted for by ferulic acid. The vast majority of phenolic acids were also found to be in the bound form⁽²⁰⁾. It has been reported that following ferulic and *p*-coumaric acids, the next most abundant phenolic acids in BSG were found to be sinapic, caffeic and

Table 2. The percentage (% dry weight) of bound phenolics, ferulic acid and *p*-coumaric acid present in brewers' spent grain

Study	Percentage (as % dry matter)		
	Bound phenolics	Ferulic acid	<i>p</i> -Coumaric acid
Bartolome ⁽³⁾	–	~0.2	~0.1
Mandalari ⁽¹⁸⁾	–	0.6	0.3
Athanasios ⁽¹⁹⁾	–	0.2–0.3	0.1–0.2
Forsell ⁽²⁰⁾	–	0.5	0.5
Treimo ⁽¹⁶⁾	0.7	–	–
Robertson ⁽²¹⁾	0.8	–	–
Szwajgier ⁽²²⁾	–	0.3	0.1

syringic acids⁽²²⁾. A summary of the phenolic acids present in BSG is given in Table 3.

Extraction of phenolic acids from brewers' spent grain

Numerous studies have been conducted to extract phenolic acids from BSG. Novel techniques for extraction, such as a rapid microwave-assisted derivatisation process have been investigated⁽¹⁹⁾. However, the majority of approaches use the basis of either acid hydrolysis or saponification (with 1–4 M NaOH) and liquid–liquid or liquid–solid extraction. A review of methods of extracting, separating and detecting phenolic acids in natural plant foods showed that the most frequently used methods involve acid hydrolysis and saponification⁽²⁴⁾. Extraction usually entails the use of solvents such as methanol and ethyl acetate. TLC is extensively used for detecting phenolic acids due to its high sample throughput. However, using HPLC gives a greater degree of separation of compounds and is highly reproducible where quantification is possible. Therefore, reverse phase-HPLC is predominantly used, but HPLC coupled with UV or diode array detection is also an option⁽²⁵⁾. A review looking at the extraction and quantification of phenolics in foods also reported that methanolic extraction and alkaline hydrolysis are commonly used for phenolic acid extraction, while a sequential alkaline hydrolysis releases bound phenolics⁽²⁶⁾. A new method has recently been developed and validated, for the release of phenolic acids (both free and bound) from cereals including barley. This method uses solid-phase extraction coupled with HPLC-diode array detection analysis and is simple, inexpensive and gives good recoveries and precision⁽²⁷⁾. Recently published results show that exogenous ferulic acid esterase produced by the probiotic organism *Lactobacillus acidophilus* K1 can successfully release the free phenolics from BSG⁽²⁸⁾. In 2005, a study looking at the hydroxycinnamate content of BSG fractions utilised saponification with 4 M NaOH. The supernatants were then neutralised and extracted with ethyl acetate, dried and re-suspended in MeOH:H₂O⁽¹⁸⁾. More recent research also used this method, giving comparable results⁽²¹⁾. Using a LUNA C18 reverse phase-HPLC column, both studies found that ferulic acid was the phenolic acid in greatest abundance in BSG, with coumaric acid being second highest. Saponification (involving the treatment of samples

Table 3. The most abundant phenolic acids present in brewers' spent grain modified from Szwajgier *et al.*⁽²²⁾

Phenolic acid	Free acid concentration (mg/100 g dry matter)	
	Mean	SE
Ferulic acid	336.3	16.0
<i>p</i> -Coumaric acid	64.4	4.6
Sinapic acid	42.0	1.1
Caffeic acid	9.9	0.7
Syringic acid	6.5	0.1
4-OH-benzoic acid	1.2	0.6
Chlorogenic acid	0.6	0.2
Protocatechuic acid	0.5	0.1

with 1–4 M NaOH solution) has been widely used to extract hydroxycinnamic acids from BSG^(3,23,29).

Extraction methods similar to those used for BSG, have also been utilised with other materials such as wheat bran extracts⁽³⁰⁾ and apple waste extracts⁽³¹⁾.

Potential health benefits of phenolic component of brewers' spent grain

As previously mentioned, ferulic and *p*-coumaric acids are the phenolic acids at highest concentrations in BSG (Table 3). A lot of research has been conducted looking at the antioxidant activity of hydroxycinnamic acids, particularly ferulic and *p*-coumaric acids. A commonly used method for quantification of antioxidant activity is the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, which measures the ability of the test compound to scavenge the DPPH radical. Chen and Ho⁽³²⁾ have shown the antioxidant potential of ferulic acid using the DPPH and Rancimat methods, but ferulic acid was a less potent antioxidant than caffeic acid and α -tocopherol⁽³³⁾. Caffeic acid has been shown to act as an antioxidant *in vitro* and scavenged radicals including DPPH and the superoxide anion⁽³⁴⁾. It has also been shown, using the DPPH assay, that a number of hydroxycinnamic acids act as antioxidants, scavenging DPPH in the order caffeic acid > sinapic acid = ferulic acid > ferulates > *p*-coumaric acid⁽³⁵⁾. Similarly, but using an alternative method, a study investigating the phenolic compounds in wheat bran extract and their antioxidant activity again found that ferulic acid was one of the strongest antioxidants using the β -carotene linoleic acid model system. The β -carotene linoleic acid model system assay is based on the principle that at a high temperature the oxidation of linoleic acid produces peroxides that decolourise β -carotene. The wheat bran extracts with highest ferulic acid concentrations (following alkaline hydrolysis) also exhibited higher antioxidant activity⁽³⁰⁾. Ferulic acid and caffeic acid have been reported to have excellent antioxidant potential at low concentrations, with the ability to scavenge a range of free radicals. Both phenolic acids scavenge the reactive oxygen species and reactive nitrogen species, with concentration-dependent scavenging of NO, superoxide and 2,20-azino-bis-3-ethyl-benzthiazoline-6-sulfonic acid radical. In line with the previously discussed evidence, caffeic acid was a stronger scavenger of the DPPH radical, but ferulic acid was better

at scavenging 2,20-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid radical and NO⁽³⁶⁾. In a recent study on beers, a direct correlation was found between ferric-reducing antioxidant power and a number of phenolic acids including ferulic, *p*-coumaric, caffeic, sinapic and vanillic acids⁽³⁷⁾. A second study also showed that some of the phenolic acids present in beer correlate with the antioxidant activity measured by the DPPH radical and superoxide anion scavenging, metal chelation and reducing power; these include syringic and caffeic acids⁽³⁸⁾. Since ferulic acid is so well recognised as an antioxidant, it is approved for use as a food additive in some countries to prevent oxidation^(39,40). In addition, it is important to note that while phenolic compounds can have an antioxidant effect, they have also been shown to act as pro-oxidants under certain conditions, thus inducing oxidative stress. Recent literature suggests that at low concentrations, many phenolics exhibit pro-oxidant behaviour, whereas the synthetic antioxidants, including α -tocopherol, do not⁽⁴¹⁾. For caffeic acid and ferulic acid to act as pro-oxidants, higher concentrations are required⁽³⁶⁾. It has also been found by using the comet assay that at high concentrations *p*-coumaric acid enhanced DNA breakage induced by H₂O₂. This may be due to the production of reactive oxygen species by *p*-coumaric acid as a result of its pro-oxidant activity⁽⁴²⁾. It has been suggested that this pro-oxidant effect is related to the presence of metal ions in the body (for example due to tissue injury releasing Fe and Cu) and is of relevance for the bioactivity of phenolic compounds *in vivo*⁽⁴³⁾. It is clear from a small number of *in vivo* studies that the hydroxycinnamic acids have antioxidant properties. Such studies are essential to understand the biological role of these phenolic acids⁽⁴⁴⁾.

In addition to their antioxidant potential, there is increasing evidence to suggest that phenolic acids can have an anti-carcinogenic effect. Caffeic acid exhibits an anti-proliferative effect on several cancer cells including mammary gland, adenocarcinoma, lymphoblastic leukaemia⁽⁴⁵⁾ and cervical cancer cell lines as assessed using the MTT assay^(45,46). The cyclooxygenase-isoform 2 (COX-2) assay has been used for determination of the anti-cancer potential of these compounds. Overexpression of COX-2 increases the conversion of arachidonic acid to prostaglandins, which are important mediators of inflammation, and are associated with cancer. Phenolic acids including caffeic acid⁽⁴⁷⁾ and vanillic acid⁽⁴⁸⁾, and polyphenols including epigallocatechin-3-gallate⁽⁴⁹⁾ and quercetin⁽⁵⁰⁾, have been shown to inhibit the expression of COX-2, possibly reducing cancer risk. Apoptosis in cancer cell lines is also an indicator of anti-carcinogenic potential and can be assessed by a number of methods including DNA fragmentation and the Hoechst staining assay. Cinnamic acid derivatives induced apoptosis in human leukaemia (HL60) and colon cancer (SW480) cell lines, as measured by the aforementioned apoptosis methods⁽⁵¹⁾. In addition, the anti-apoptotic effect of phenolic compounds including ferulic acid and caffeic acid on human peripheral blood mononuclear cells was investigated⁽⁵²⁾. Caffeic acid inhibited externalisation of phosphatidyl serine, which indicates the pre-apoptotic stages, and hence it was concluded to have an anti-apoptotic effect. DNA

fragmentation was analysed using an apoptotic DNA ladder kit. It was shown that pre-treating cells with caffeic acid, ferulic acid or ellagic acid before exposure to H₂O₂ inhibited DNA fragmentation. Recently published data add to the evidence available in the area, by measuring the ability of phenolic compounds to modulate NF- κ B activity. In the inflammatory process, NF- κ B is a transcription factor, whose increased activation has been reported in several human cancers⁽⁵³⁾. Free phenolic acids that can be found in cereal grains (including ferulic, caffeic, sinapic and *p*-coumaric acids) significantly modulate NF- κ B activity in U9373 κ B-LUC cells, with a desired level of modulation being achieved by the synergistic action of phenolic acids and other phenolic compounds⁽⁵⁴⁾. Animal studies have also been carried out to determine the anti-carcinogenic potential of phenolic acids. An animal study to establish the effect of curcumin, chlorogenic acid, ferulic acid and caffeic acid on tumour promotion in the skin of mice showed that chlorogenic, ferulic and caffeic acid prevented the number of 12-*O*-tetradecanoylphorbol-13-acetate-induced tumours per mouse by 60, 28 and 35%, respectively⁽⁵⁵⁾. Results of a later animal study suggest that ferulic acid not only inhibits the growth of aberrant crypt foci in the colon but also prevents the conversion of pre-neoplasia to malignant neoplasia⁽⁵⁶⁾. A recently published review on plant phenolics reported that natural phenolics, including tea and fruit polyphenols, play an antagonistic role in all stages of cancer development and that further study on these compounds will provide information regarding their possible future pharmaceutical use⁽⁵⁷⁾.

Cytokines are small cell signalling molecules involved in the inflammatory response, these include interleukins and interferons (for example interferon- γ). The ability of a compound to alter the production of a stimulated cytokine or NO indicates the compound's potential to act as an immune-modulator. Murakami *et al.*⁽⁵⁸⁾ investigated the effect of both ferulic acid (from rice bran) and FA15 (a derivative of ferulic acid) on NO synthase, COX-2 and TNF α in the RAW264-7 murine macrophage cell line. Unlike the ferulic acid isolated from rice bran, the synthesised FA15 derivative was found to inhibit the release of TNF α and reduce the protein expression of both nitric oxide synthase and COX-2⁽⁵⁸⁾. Ferulic acid has also been shown to inhibit macrophage inflammatory protein-2 and TNF α production, induced by lipopolysaccharide in a macrophage cell line. The effect, although dose-dependent, was very weak compared with the effect of dexamethasone (a well-known inhibitor of interleukins)⁽⁵⁹⁾. In Japanese Oriental medicines, *Cimicifuga heracleifolia* is often used as an anti-inflammatory drug. Ferulic acid has been shown to be among the main phenolic acids in *C. heracleifolia*⁽⁶⁰⁾. Sakai *et al.*⁽⁶¹⁾ showed that ferulic acid and isoferulic acid could reduce macrophage inflammatory protein-2 production in a dose-dependent manner in RAW264-7 cells. It was suggested that ferulic acid and isoferulic acid are responsible, at least in part, for the anti-inflammatory properties of the *C. heracleifolia* drug⁽⁶¹⁾. Recently published data have shown that ferulic acid and *p*-coumaric acid inhibited lipopolysaccharide-induced NO production and inducible NO synthase in macrophages⁽⁶²⁾. This supports earlier evidence suggesting that these phenolic

Table 4. DNA damage in U937 cells following 24 h incubation with 0.5% (v/v) pale (P1–P4) or black (B1–B4) brewers' spent grain phenolic extracts or 0.1 µg/ml ferulic acid (adapted from McCarthy *et al.*⁽⁶⁹⁾)

Sample	DNA damage (% tail DNA) oxidant – H ₂ O ₂		DNA damage (% tail DNA) oxidant – SIN-1		DNA damage (% tail DNA) oxidant – 4-NQO		DNA damage (% tail DNA) oxidant – <i>t</i> -BOOH	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	3.2 [†]	0.5	4.3 [†]	0.4	3.1 [†]	0.3	4.7 [†]	0.6
Oxidant control	40.3 [†]	0.3	40.0 [†]	3.9	39.5 [†]	1.1	15.5 [†]	0.8
P1	30.3 [†]	7.3	32.8 [†]	4.9	39.0 [†]	6.8	14.5 [†]	0.8
P2	29.0 [†]	6.0	22.7 ^{*†}	1.9	40.7 [†]	4.3	22.6 [†]	3.0
P3	39.9 [†]	3.9	33.8 [†]	2.9	37.3 [†]	3.2	22.1 [†]	3.1
P4	23.1 [†]	3.2	32.2 [†]	6.1	37.8 [†]	3.6	17.4 [†]	2.6
B1	25.8 ^{*†}	7.5	33.6 [†]	2.9	36.1 [†]	5.7	13.6 [†]	0.9
B2	9.6 ^{*†}	1.1	6.9 ^{*†}	1.0	31.9 [†]	3.2	18.5 [†]	3.3
B3	16.0 ^{*†}	2.0	18.1 ^{*†}	0.3	33.4 [†]	2.4	16.6 [†]	1.8
B4	14.0 ^{*†}	3.8	24.1 ^{*†}	2.2	33.7 [†]	6.8	14.0 [†]	2.7
Ferulic acid	17.1 ^{*†}	0.8	30.8 [†]	3.0	32.6 [†]	5.2	11.0 [†]	1.9

SIN-1, 3-morpholinosydnonimine hydrochloride; 4-NQO, 4-nitroquinoline oxide; *t*-BOOH, *tert*-butylhydroperoxide.

[†]Data represent the mean of at least three independent experiments.

[†]Denotes a significant difference in DNA damage ($P < 0.05$), relative to oxidant control. Statistical analysis by ANOVA followed by Dunnett's test.

acids can act as anti-inflammatory agents, by reducing TNF α induced IL-6 production in adipocytes. Quercetin, *p*-coumaric acid and resveratrol showed greatest inhibition of IL-6 production⁽⁶³⁾.

Oxidised LDL is a well-recognised risk marker of CVD which is principally caused by atherosclerosis⁽⁶⁴⁾. Evidence exists for the effect of hydroxycinnamic acids on the inhibition of LDL oxidation. Nardini *et al.*⁽⁶⁵⁾ demonstrated the antioxidant effect of hydroxycinnamic acid derivatives such as caffeic, ferulic and *p*-coumaric acids on LDL oxidation *in vitro*, with the use of the copper ion Cu²⁺ as a catalyst. At a concentration of 100 µM, all phenolic acids except *p*-coumaric acid inhibited LDL oxidation; at 20 µM, ferulic acid inhibited about 92% of Cu-catalysed human LDL oxidation; at 5 µM only caffeic acid strongly inhibited the oxidation of LDL⁽⁶⁵⁾. A second study using similar methodology also found that both ferulic and *p*-coumaric acid showed a dose-dependent inhibition of human LDL oxidation *in vitro* when tested at 5, 10 and 20 µM⁽⁶⁶⁾. In agreement with these data, results of a later study showed sinapic, ferulic and *p*-coumaric acids inhibited LDL oxidation⁽⁶⁷⁾. Other compounds that have shown the ability to reduce Cu-induced LDL oxidation include catechin⁽⁶⁸⁾.

Our research group is the first, to our knowledge, to look specifically at phenolic extracts from BSG. Four extracts from pale BSG (P1, P2, P3 and P4) and four extracts from black BSG (where the grain is roasted to 200°C before brewing; B1, B2, B3 and B4) were analysed. Each extract results from a different step in the extraction process and hence contains different phenolic acid compositions. Extract 1 (P1, B1) contains free phenolics, extract 2 (P2, B2) contains bound phenolics, extract 3 (P3, B3) contains the remainder of bound phenolics and extract 4 (P4, B4) contains phenolics extracted with 110 mM NaOH⁽⁶⁹⁾. The ability of the phenolic extracts to protect against oxidant-induced DNA damage was determined using the comet assay. In the U937 cell line, oxidative DNA damage was

induced by a range of oxidants; H₂O₂, 3-morpholinosydnonimine hydrochloride, 4-nitroquinoline oxide and *tert*-butylhydroperoxide. Table 4 shows the ability of the extracts to protect the cells against DNA damage. There was no protection against DNA damage induced by either 4-nitroquinoline oxide or *tert*-butylhydroperoxide. Ferulic acid and the black BSG extracts significantly reduced the DNA damage induced by H₂O₂, while P2, B2, B3 and B4 significantly reduced the percent tail DNA induced by 3-morpholinosydnonimine hydrochloride⁽⁶⁹⁾. The four oxidants used have different mechanisms of action; damage induced by both H₂O₂ and 3-morpholinosydnonimine hydrochloride involve the Fenton reaction which is an Fe-dependent reaction, 4-nitroquinoline oxide mimics the action of UV and Cu²⁺ plays an essential role, whereas Fe does not⁽⁷⁰⁾, *tert*-butylhydroperoxide causes lipid peroxidation and acts in a Ca²⁺-dependent manner and Fe plays less of a role than in H₂O₂-induced damage⁽⁷¹⁾. Therefore, BSG phenolic extracts may provide protection against oxidant-induced DNA damage by Fe chelation⁽⁶⁹⁾.

In summary, there is increasing evidence to suggest that phenolic acids, including those found at highest concentrations in BSG, can confer potential health benefits including anti-inflammatory, antioxidant, anti-carcinogenic and anti-atherogenic effects. Recent data suggest that BSG has antioxidant potential and therefore further research on the phenolic compounds extracted from BSG is warranted.

Incorporation of brewers' spent grain into feed/foodstuffs

Animal food

As previously mentioned, BSG contains approximately 20 and 70% protein and fibre, respectively, and it is due to this favourable chemical composition that it has great potential for use as a raw material/food ingredient⁽⁴⁾. BSG is an ingredient of significant importance for ruminants.

When administered with low-cost N sources such as urea, BSG can supply all the essential amino acids to the ruminants. The effect of BSG on milk yield and composition and the blood components of dairy cattle has also been studied⁽⁷²⁾. The cattle received a diet consisting of ground maize, maize silage, soya bean meal and wheat bran, with the latter three being substituted with wet brewer's grain in the treatment group. The study showed the treatment group had an increased milk yield, milkfat and milk total solids content. Blood components such as glucose, cholesterol, Na and TAG were not affected. While the main outlet for BSG is currently as a feedstuff for dairy cattle, research has also been conducted looking at the benefits of BSG for use as a feed for poultry and fish. The effect of replacing rice bran in a fish diet with 10–40% brewery waste grains has been investigated⁽⁷³⁾. The brewery waste used contained 19% crude protein, 18–20% crude fibre and had a good amino acid profile. It was found that carp (oily freshwater fish) had better growth performance on diets containing brewery waste than the control group. The authors attributed this enhanced growth performance to the high-quality protein contained in the waste grains. A more recent study showed that biodegraded BSG contained cysteine, lysine and methionine in addition to fourteen other amino acids⁽⁷⁴⁾. Depending on the microbe used to degrade the BSG, different amino acid concentrations were found, with alanine consistently at highest concentrations. This composition was noted to be of particular importance for poultry as cysteine, lysine and methionine are the main amino acids required in poultry nutrition. For convenience, the possibility of producing dry BSG cakes suitable for long-term storage was examined using membrane filter press technology⁽¹³⁾. These dry cakes could be used as an animal feed at any time, or as a starting material for the production of other products using BSG.

In summary, evidence suggests that whole BSG, fed as part of a total mixed ration, has many nutritional benefits for a range of animals, particularly dairy cattle. This has resulted in the routine use of BSG as an animal feed for cattle.

Human food

In addition to its use as an animal feed, BSG has been incorporated into foodstuffs for human consumption. Given its low cost and high nutritional value, BSG makes an ideal ingredient for human foods such as biscuits and ready-to-eat snacks, particularly where there is a need to increase fibre content. In 1978, the possibility of preparing cookies with flour containing BSG at levels ranging from 5 to 60% was examined⁽⁸⁾. At 40% BSG addition, the physical qualities of the cookies were sustained. This supplementation level gave a 74% increase in N and increased crude fibre ten-fold. These results were supported by work published in 2002, where the authors looked at the effect of BSG (at levels of 5–25%) on the fibre content and quality of cookies⁽⁷⁵⁾. As the level of addition of BSG increased, there was a significant increase in dietary fibre content. Another documented foodstuff suitable for the inclusion of BSG is ready-to-eat snacks⁽¹⁾. BSG was added to the formulation mix (consisting of ingredients such as corn starch and wheat flour) at levels

ranging from 10 to 30%. The incorporation was successful, increasing dietary fibre and crude protein levels. Similarly, the addition of BSG into extruded snack food has been studied⁽⁷⁶⁾. The maize flour of chickpea snacks was replaced with BSG at levels of 10, 20, 25 and 30%. The parameters measured included the effect of BSG supplementation on texture, colour, moisture, fat, fibre, starch, protein, phenolic compounds and antioxidant capacity. With increasing levels of BSG addition, the percent protein, fat and fibre content increased, while starch decreased. It was suggested that foods fortified with BSG be considered as functional foods. In a further study by Stojceska and Ainsworth⁽⁷⁷⁾, BSG was incorporated at different levels (0–30%) into wheat flour breads treated with four different enzymes and the bread quality was then evaluated⁽⁷⁷⁾. Similar to the previous study, it was found that the fibre content of the breads was significantly increased by BSG addition. The change in fat content was significantly linked to the addition of BSG. When addition of BSG is combined with the appropriate use of enzymes, the shelf life, texture and loaf volume can also be improved. Initially, it was thought that BSG was too granular for direct addition to food and that it would have to first be converted to flour before use. However, a study in 2009 demonstrated that BSG of various particle sizes could be effectively used in the production of frankfurters⁽⁷⁸⁾. The control frankfurter had the highest score for acceptability, but the other products also had high scores, with the score decreasing with increase in particle size and reduction in fat levels. The authors suggested that BSG be used to produce low-fat high-fibre meat products. In addition to particle size, there are a number of points for consideration with the incorporation of BSG into foodstuffs. Firstly, there are concerns about appearance. When moist, BSG is brown in colour, thus it can only be effectively integrated into off-white products. Such foods include cookies and cakes. More importantly, it is imperative that the organoleptic properties of the foodstuff remain acceptable to consumers and are similar to the current commercially available products. The study by Prentice *et al.*⁽⁸⁾, demonstrated that BSG addition at a level of 15% was the upper limit for organoleptic acceptability⁽⁸⁾. At this level, the organoleptic quality was lowered but still remained acceptable to consumer panels. Similarly, Stojceska *et al.*⁽¹⁾ found that there was a limit to acceptability⁽¹⁾. At a level of addition of 30%, physicochemical characteristics (such as texture, colour and hardness) remained acceptable. However, the authors concluded that addition of BSG at 20% level was optimal to maintain properties of commercially available snack foods. Where the protein hydrolysates are to be extracted and incorporated into foodstuffs, there is concern over the bitter taste of some peptides, due to the hydrophobic amino acid content⁽⁷⁹⁾.

Conclusion

The literature shows that phenolic compounds including ferulic, caffeic and *p*-coumaric acid can have antioxidant, anti-cancer, anti-atherogenic and anti-inflammatory effects. Given that these phenolic acids are some of the major phenolics in BSG, it is expected that phenolic extracts

from spent grain may also exhibit similar properties and have the potential to be developed for a range of bioactivities. BSG currently functions as an animal feed, having many nutritional benefits. While some attempts have been made to incorporate the bioactive components of BSG into foodstuffs, further research in this area is needed. Given the potential bioactive nature of the phenolic extracts from BSG, and the large amounts of BSG produced annually as a low value co-product, it is imperative that an alternative use be explored.

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Article

Protein Hydrolysates from Agricultural Crops—Bioactivity and Potential for Functional Food Development

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Abstract: There has been an unprecedented demand for inexpensive plant-derived protein hydrolysates in recent years, owing to their potential nutritional applications. This review examines existing evidence regarding protein hydrolysates from agricultural crops such as wheat, soy, rapeseed, sunflower and barley. The bioactivity of these protein hydrolysates, including antioxidant and anti-inflammatory capabilities are discussed. In addition to evidence regarding their potential to enhance human nutrition, the effect of the hydrolysates on the techno-functional properties of foods will be reviewed.

Keywords: plant-derived; protein hydrolysates; bioactive; techno-functional

1. Introduction

Humans require a protein intake sufficient to maintain the body nitrogen balance and allow for desirable rates of deposition during growth and pregnancy. Ingestion of protein amounts greater than requirements results in the excess being metabolised and excreted. Conversely, in the case of inadequate dietary protein intake, the body utilizes its own proteins as a source of nitrogen; therefore a regular and sufficient intake is essential. Protein performs a number of key functions in the body including the building and repair of tissues, cell signalling and the provision of energy (4 kcal/g protein). Proteins also perform enzymatic and structural functions.

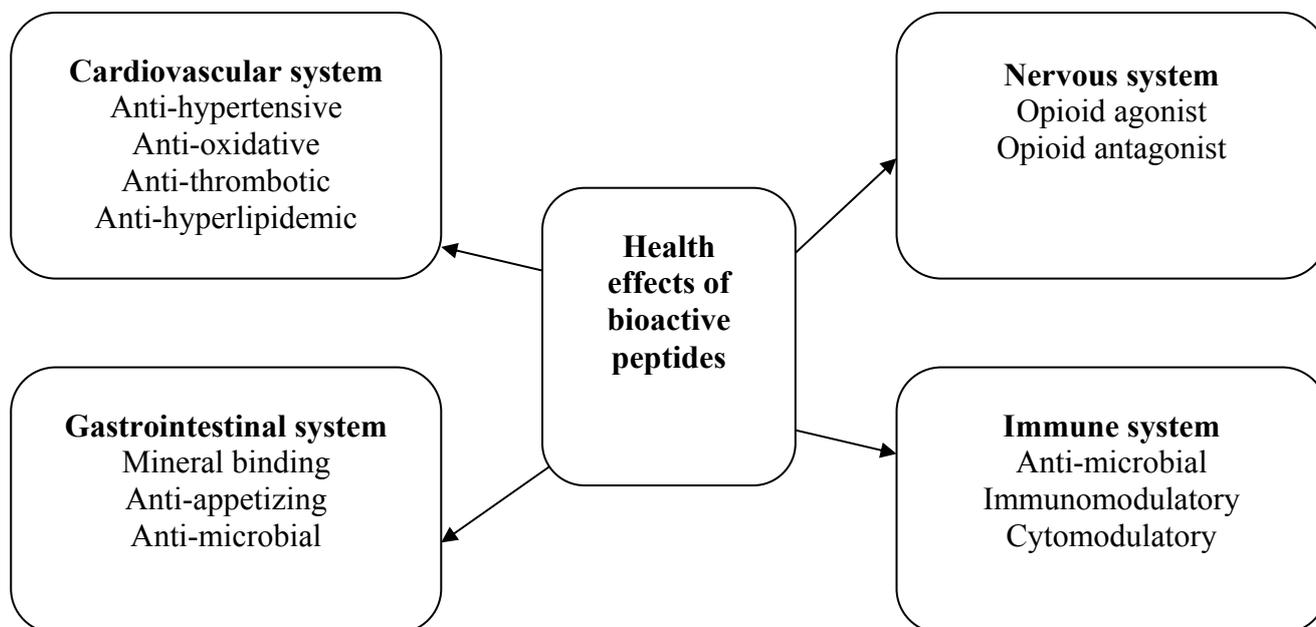
Protein hydrolysates have been defined as “mixtures of polypeptides, oligopeptides and amino acids that are manufactured from protein sources using partial hydrolysis” [1]. There has been growing

interest in these preparations over the last two decades, with novel bioactive peptides continually being discovered, as it has been shown that short-chain peptides from hydrolyzed proteins have a higher nutritive value and may be utilized more efficiently than an equivalent mixture of free amino acids [2]. Milk-based products are the source of the greatest number of bioactive peptides isolated to date. Other sources include meat, eggs and fish, in addition to plant sources such as soy and wheat [3]. Figure 1 illustrates the effect of bioactive peptides on major body systems. It has emerged that protein hydrolysates have many uses in human nutrition; ingredients in energy drinks, weight-control and sports nutrition products [4], sources of nutrition for elderly and immuno-compromised patients [5]. Clinical applications have also been suggested, including treatment of Phenylketonuria (PKU), liver disease, Crohn’s disease and ulcerative colitis [6]. Other functions of plant-derived protein hydrolysates have been discussed in detail elsewhere [7]. These include use as natural herbicides, particularly corn gluten meal and soy and wheat hydrolysates [8] and as replacements for materials of bovine origin in fermentation media, to reduce risk of Bovine Spongiform Encephalopathy (BSE) contamination [9].

In recent years there has been an unprecedented demand by both consumers and industry, for inexpensive plant-derived proteins and bioactive peptides for human consumption. Additionally, alternative uses for co-products of the plant processing industry are highly sought. Such co-products include brewers’ spent grain (BSG), wheat bran and okara (a soybean by-product of tofu production), which are excellent sources of both protein and fibre [10,11].

The present review focuses on the bioactivity of protein hydrolysates from a range of agricultural crops, and their potential for inclusion into functional foods.

Figure 1. Physiological effects of food derived bioactive proteins on major body systems.



2. Preparation of Protein Hydrolysates

2.1. Protein Hydrolysis

Hydrolysis of proteins involves the cleavage of peptide bonds to give peptides of varying sizes and amino acid composition. There are a number of types of hydrolysis; enzymatic, acid or alkali hydrolysis. Chemical hydrolysis is difficult to control and reduces the nutritional quality of products [12], destroying L-form amino acids and producing toxic substances such as lysino-alanine [13]. Enzymatic hydrolysis works without destructing amino acids and by avoiding the extreme temperatures and pH levels required for chemical hydrolysis, the nutritional properties of the protein hydrolysates remain largely unaffected [12]. Production of protein hydrolysates in the food industry involves the use of digestive proteolytic enzymes from animals including chymotrypsin, trypsin and pepsin, or food grade enzymes obtained from plants and microorganisms which are regarded safe for human nutrition. Following protein hydrolysis, fractions can be categorised according to two characteristics. The first category consists of fractions with a high amino acid content. The second category consists of bioactive peptides with an amino acid sequence which is inactive in the intact protein molecule but becomes active in the hydrolysate following exposure to digestive and/or proteolytic enzymes [1]. It is worthy to note that the process of manufacturing protein hydrolysates has essentially remained the same since its emergence several decades ago and is still in its infancy [14]. Co-operation between manufacturers and end-users is necessary to develop optimum hydrolysates for specific functions [14].

2.2. Post-Hydrolysis Treatment

Following hydrolysis, the “crude hydrolysates” may undergo further processing. Commonly used post-hydrolysis processes include heat inactivation, ultrafiltration, hydrolysis by exoproteases and treatment with specific enzymes. Table 1 details the main post-hydrolysis processes and the function of each of these processes. Control of the molecular size of protein hydrolysates is an essential step in the development of protein hydrolysates for dietary use. Removal of high molecular weight proteins and peptides is primarily carried out using ultrafiltration. Protein hydrolysates can have a bitter taste and the elimination or reduction of this bitterness is essential to make the hydrolysates acceptable to consumers. The bitterness of protein hydrolysates is attributable to their hydrophobic amino acid content [1] and the release of these amino acids by exoproteases can reduce bitterness [15]. Post-hydrolysis processes can also be used to produce hydrolysates for the treatment of clinical conditions. For example, the use of phenylalanine ammonia lyase enzyme can reduce the phenylalanine content in protein hydrolysates, producing a hydrolysate suitable for patients with phenylketonuria, a disorder of phenylalanine metabolism [6].

Table 1. The main processes used following hydrolysis of protein (post-hydrolysis processes).

Process	Function
Heat inactivation	Inactivation of proteolytic enzymes
Ultrafiltration	Removal of high molecular weight proteins and peptides
Use of specific enzymes	Reduce content of specific amino acids
Hydrolysis by exoproteases	Hydrolysis, reduction of bitterness
Activated carbon	Reduction of bitterness
Absorption chromatography	Reduce content of aromatic amino acids

3. Bioactivity of Protein Hydrolysates

3.1. Antioxidant

Protein hydrolysates from agricultural crops including soy, rapeseed, wheat, sunflower and barley have been investigated for their antioxidant potential. In 1980, it was reported that following proteolysis, soy protein hydrolysates showed antioxidant potential, as measured by the thiobarbituric (TBA) assay, which is a measure of lipid peroxidation. It was suggested that the release of bound antioxidant phenolics or copper chelating agents was responsible for the observed antioxidant activity [16]. Using similar methodology, Peña-Ramos and Xiong [17] provided comparable results. Soy protein hydrolysates prepared with Flavourzyme or chymotrypsin had antioxidant potential greater than unhydrolysed soy protein isolate. However, it was found that extensive degradation using enzymes such as papain unfavourably altered the antioxidant activity [17]. A further study measured the ability of these soy protein hydrolysates, prepared with Flavourzyme or chymotrypsin, to inhibit lipid oxidation in corked pork patties. However, in contrast to the initial study, the soy protein hydrolysates did not affect lipid oxidation by the TBA assay, but reduced conjugated diene (CD, a marker of free radicals) formation in stored pork patties [18]. CD is a secondary product of lipid oxidation. Later studies have also reported the antioxidant potential of soy protein hydrolysates [19–22]. Soy protein hydrolysates post-treated with ultrafiltration, resulting in low molecular weight fractions (<10 kDa) have shown greatest antioxidant potential [20].

Protein hydrolysates from a range of other agricultural crops have been less extensively studied in comparison to soy hydrolysates. Rapeseed protein hydrolysates exhibited a dose-dependent inhibition of lipid peroxidation by a speculated proton donation mechanism [23]. Later studies supported these findings, with rapeseed hydrolysates showing the ability to act as reducing agents and scavenge hydroxyl radical and superoxide anion [24,25]. By employing the post-hydrolysis process of affinity chromatography, copper chelating peptides were isolated from sunflower protein hydrolysates. The ability to chelate copper increased mineral bioavailability and exerted antioxidant effects [26,27]. Protein hydrolysates isolated from wheat germ also possess radical scavenging abilities, with an antioxidant activity close to that of the well known, antioxidant α -tocopherol. Interestingly, these hydrolysates had low molecular weight <1500 Da [28]. Similarly, enzymatic hydrolysates of buckwheat showed excellent antioxidant potential, scavenging (DPPH) radical, inhibiting linoleic acid peroxidation and possessing reducing power [29].

Protein hydrolysates isolated from co-products of the plant processing industry have also been investigated for antioxidant activity. While BSG protein isolate and associated hydrolysates do not possess antioxidant potential [30], okara hydrolysates protect against oxidation of linoleic acid [31]. Furthermore, it has been shown that fermentation of okara using *Bacillus subtilis* B2 can greatly improve its antioxidant activity, thus adding value to this co-product [32].

3.2. Anti-hypertensive

Normal blood pressure is in the range of 100–140 mmHg (systolic) and 60–90 mmHg (diastolic). Values greater than 140 mmHg (systolic) and 90 mmHg (diastolic) are classified as hypertension or high blood pressure. In hypertension, the blood pressure in the arteries is elevated and the heart has to work hard to pump blood around the body. According to the World Health Organisation, high blood pressure is particularly relevant in middle income European countries and African countries. A high percentage of stroke (51%) and ischaemic heart disease (45%) deaths worldwide are attributable to high systolic blood pressure [33]. Dietary and lifestyle changes, including a reduction in salt intake and an increase in physical activity levels, can positively influence blood pressure. However, in cases where such changes are ineffective or insufficient, drug treatments may be prescribed. Angiotensin-converting enzyme (ACE) inhibitors are an example of a drug treatment to control blood pressure. ACE reduces the conversion of angiotensin-1 (vasodilatory) to angiotensin-2 (vasoconstrictory) resulting in a reduced blood pressure. Hence, there is great interest in novel compounds that can inhibit ACE. In 2000, wheat germ hydrolysate and its dominant peptide significantly reduced mean arterial pressure (MAP) in spontaneously hypertensive rats. It was shown that the dominant bioactive peptide could be metabolised by an aminopeptidase to form an ACE inhibitory metabolite, indicating potential blood pressure lowering effects of the metabolite after absorption [34]. Similarly, a buckwheat protein hydrolysate was found to reduce systolic blood pressure in spontaneously hypertensive rats and also inhibit ACE, particularly when hydrolysis was carried out with pepsin followed by chymotrypsin and trypsin [35]. More recently, it was found that ultrasonic pre-treatment promotes the release of ACE inhibitory peptides during enzymatic proteolysis of wheat germ [36]. van der Ven *et al.* [37] described the processing conditions necessary to produce hydrolysates with maximal ACE inhibitory activity. It was suggested that the ACE inhibitory activity of protein hydrolysates is due to the synergistic action of the different peptides present, thus the isolation of peptides is not justified and optimising the entire peptide composition is essential. Response surface modelling (which comprises a body of methods to explore optimum operating conditions through experimental methods [38]) is effective in the optimisation of a number of parameters simultaneously to produce a hydrolysate with maximum ACE inhibitory activity. Similar to the results for antioxidant activity of protein hydrolysates, it was found that ACE inhibitory activity of soy protein hydrolysates increased with decreasing molecular weight of peptides; hence ACE inhibitory peptides have low molecular weight [39]. This study also focused on an important consideration for the formulation of anti-hypertensive functional foods, that is, the digestibility of the protein hydrolysates. Following *in vitro* gastric digestion, which simulates conditions in the human stomach, of the soy protein hydrolysates, the ACE inhibitory activity was retained. Stability during processing is another key factor and hydrolysates were shown to have sufficient stability to various heat (20–100 °C) and pH (pH 2–10) treatments. These findings were supported by a study published in

2006, where it was also reported that soy protein hydrolysates possessed ACE inhibitory activity that was unaffected by *in vitro* gastrointestinal proteases [40]. Protein hydrolysates from a range of other crops including potato [41], corn [42,43], spinach [44], sunflower [45,46], peanut [47] and rapeseed [48] have exhibited high ACE inhibitory activities. Rice-bran, an under-utilized co-product of rice milling also has the potential to inhibit ACE activity, high molecular weight hydrolysates (10–50 and >50 kDa) resulting in at least 50% inhibition [49]. In addition to ACE inhibition, there are a number of other potential mechanisms of inhibiting hypertension. These include activation of endothelial nitric oxide synthase (NOS), reduction of Ca^{2+} in vascular smooth muscle cells (VSMC) and rennin inhibition [50]. The ability of a compound to induce nitric oxide (NO) production via NOS and increase endothelial cell Ca^{2+} concentration contributes to vasodilation and reduced blood pressure [50]. Studies utilizing these mechanisms have also been carried out; for example soy protein isolate and hydrolysates have been shown to increase NO release in human aortic endothelial cells (HaoEC) [51].

3.3. Cardiovascular Disease

Cardiovascular diseases (CVD) are the primary cause of death globally, representing 30% of all global deaths in 2008 [52]. Cardiovascular diseases are diseases of the heart and blood vessels and include coronary heart disease, cerebrovascular disease and peripheral arterial disease [52]. An unhealthy diet and physical inactivity are among the two main risk factors for CVD, resulting in raised blood pressure, blood lipids and blood glucose, overweight and obesity, which are classed as intermediate risk factors for CVD. The consumption of vegetable protein has been associated with a lower risk of coronary heart disease, in comparison to consumption of animal protein [53–55]. This observed effect may be attributed to decreases in serum cholesterol levels [56]. For more than 100 years, animal studies have shown the cholesterol lowering effect of soy protein compared with animal protein [57]. Reduced intestinal cholesterol absorption and increased faecal bile acid excretion, reduced levels of hepatic lipogenic enzymes such as glucose-6-phosphate dehydrogenase (G6PDH) and stimulation of adiponectin, a cytokine involved in adipocyte differentiation and insulin sensitivity are all possible mechanisms for the lipid lowering effect of soy protein [58]. A meta-analysis of 38 studies indicated that soy protein consumption significantly decreased serum cholesterol, low density lipoprotein (LDL) cholesterol and triglyceride concentrations; there was also an increasing trend in high density lipoprotein (HDL) cholesterol concentrations [59]. An animal study carried out on genetically obese mice and dietary obese rats measured the effect of soy protein isolate and hydrolysate and on the rate of body fat disappearance. Feeding with either soy isolate or hydrolysate resulted in a significantly reduced body-fat content and plasma glucose levels in comparison with control, casein fed rodents. A decrease in the plasma total cholesterol level was also observed [60]. Soy hydrolysates have also demonstrated effects such as decreased cholesterol absorption both *in vitro* and in rats [61], anti-adipogenic *in vitro* [62], reduced fat mass and serum lipid in rats [63]. Sunflower hydrolysates produced using alcalase or pepsin inhibited the incorporation of cholesterol into bile salts micellar suspensions [64]. As mentioned in section 3.2 *in vitro* digestion did not decrease ACE inhibitory bioactivity of soy protein hydrolysates. It has also been shown that digestion of sunflower protein hydrolysates with simulated intestinal fluids produces new peptides that inhibit cholesterol incorporation into micellar suspensions [64]. Protein hydrolysates from crop processing co-products

have been less extensively studied, but rice bran hydrolysates have demonstrated hypocholesterolemic activity, by reducing total cholesterol and increasing HDL cholesterol in male Wistar rats [65].

3.4. Exercise and Performance Enhancement

Muscle glycogen is an important fuel during periods of prolonged exercise and a relationship between increasing exercise intensity and a reliance on muscle glycogen is evident. Fatigue has been directly related to depleting glycogen stores [66–68]. Hence, the post-exercise glycogen synthesis rate is essential in determining the time required for recovery. It has been shown that carbohydrate and wheat protein hydrolysate combined with an amino acid mixture (0.8 g/kg/hr and 0.4 g/kg/hr, respectively), administered to cyclists for a five hour period post-exercise, increases glycogen synthesis rates compared to administration of carbohydrate alone [69]. Similarly, peak creatine kinase (CK) levels, as a result of initial muscle injury, were suppressed by wheat gluten hydrolysate in a dose-dependent manner *in vivo* [70]. Soy protein isolate is beneficial in muscle protein synthesis (MPS) following exercise. Consumption of the soy protein isolate was more effective than casein isolate but less effective than whey hydrolysate at increasing MPS both at rest and following resistance exercise [71]. Calbet and MacLean [72] suggested that carbohydrate and protein hydrolysates stimulate a synergistic insulin response, regardless of protein source. It was also found that the glucagon response depends on the increase in plasma amino acid composition, following protein solution ingestions and that pea and whey protein hydrolysates increased insulin to a greater extent and increased plasma amino acids more rapidly than cow's milk solution. It has been suggested that hydrolysates, particularly containing di- and tri-peptides, are absorbed more rapidly than either intact proteins or free form amino acids [73] which would support the use of protein hydrolysates for post-exercise recovery drinks as this would result in a greater increase in plasma amino acid concentration compared with the intact protein, over a two hour period [74]. The concentration of amino acids present in the blood regulates protein synthesis [75].

3.5. Other Clinical Applications

Protein hydrolysates represent an alternative to intact proteins and elemental (amino-acid based) formulas for the treatment of patients with various conditions. Phenylketonuria is a disorder of amino acid metabolism, specifically, absence or deficiency of phenylalanine hydroxylase for the conversion of phenylalanine to tyrosine. The lack of this enzyme results in phenylpyruvic acid accumulation in the blood which has intellectual and neurological implications if left untreated. Protein hydrolysates free of phenylalanine have been used for the treatment of phenylketonuric infants, with positive results on physical growth and mental development [76,77]. Hydrolysates suitable for the treatment of phenylketonuria have been prepared from animal proteins including casein [78] and whey [79]. Plant protein hydrolysates low in phenylalanine have been studied to a lesser extent; however the potential of a low-phenylalanine soybean hydrolysate for dietetic purposes has been investigated [80]. In patients with chronic liver disease, complex alterations in the metabolism of proteins occurs and nutritional support is essential in the pathogenesis and treatment of this disease [6]. Patients with chronic liver failure have high plasma levels of aromatic amino acids (AAA) and methionine and low levels of branched-chain amino acids (BCAA) [81,82]. While casein hydrolysates are commonly used

for nutritional applications in patients with chronic liver disease, a protein source with a higher level of BCAA is more desirable. Sunflower globulins have been suggested as excellent protein sources for the development of protein hydrolysates with high levels of BCAA [83]. Sunflower protein hydrolysate is recommended for enteral, parenteral and oral nutrition of liver disease patients, being hypoallergenic, having low bitterness and providing a high Fischer ratio (BCAA:AAA) of approximately 75 [84].

3.6. Further Uses

Advances in the understanding of protein hydrolysates have resulted in their use in biotechnology and fermentation. Hydrolysates have the ability to increase both production of monoclonal antibodies and productivity of therapeutic drugs produced by microorganisms and animal cells [7]. However, the most basic function of protein hydrolysates in biotechnology is as a source of nitrogen in industrial fermentation, cell culture and microbiological media [14]. As a protective mechanism against the spread of BSE from bovine animals to humans, plant materials have been recommended as an alternative for inclusion into fermentation media. Tryptone (a digest of casein) in Luria-Bertani broth supplies essential growth factors for *Escherichia coli* (*E. coli*). It has been shown that non-bovine and plant hydrolysates are efficient replacements for tryptone, measured by growth rate and growth yield of *E. coli* [9]. Furthermore, an animal-free cell culture medium supplement has been developed that can improve the bio-performance of the culture medium by providing peptides, carbohydrates, lipids, vitamins and minerals. [85]. Plant protein hydrolysates have also proved useful in the area of weed control. Corn gluten meal is commercially available as a natural pesticide, with corn gluten hydrolysate being suggested for similar results with easier application as a spray [86]. Corn, soybean and wheat protein hydrolysates have also been developed as herbicides [8].

4. Techno-Functional Properties of Protein Hydrolysates

4.1. Solubility

Solubility is the most important and generally the first techno-functional property examined during the development of new protein ingredients [87] due to its considerable effect on other techno-functional properties [88,89]. It has been proposed that reduction of the secondary structure of a protein and the enzymatic release of smaller polypeptide units are responsible for the increased solubility of hydrolysates compared to the original intact protein [90,91]. The solubility of a number of protein hydrolysates from agricultural crops has been studied. Barley protein hydrolysates showed highest solubility at strongly basic (pH 10–pH 12) conditions [92]. Similarly, Claver *et al.* [93] found that the solubility of wheat protein hydrolysates was strongly influenced by pH, with lowest and highest solubility at pH 4 and 6, respectively. The use of rapeseed and other oilseed protein isolates is restricted due to their low solubility, which is a result of protein denaturation during industrial oil extraction [94]. To improve solubility and functionality, protein isolates from oilseeds can be hydrolysed. It has been found that rapeseed hydrolysates exhibit >90% solubility at pH 5–9 [95]. Soy protein hydrolysates were found to be almost completely soluble (>99%) in the range of pH 2–9, whereas the intact protein had highest solubility at pH 9 [96]. Tsumara *et al.* [97] also demonstrated that the solubility of soy protein hydrolysates was pH-dependent. The production of hydrolysates that

are soluble at acidic pH is essential for the supplementation of fruit juices and acidic drinks [6,98]. In contrast to soy protein hydrolysates, okara protein isolates showed highest solubility at pH 12, with acid modified isolates enhancing solubility, thus increasing potential applications of okara protein as a food ingredient [99]. Chan and Ma [99] suggested that the low solubility of okara (a by-product of soymilk manufacture) hydrolysates compared to soy hydrolysates is due to protein denaturation caused by severe heat treatments during soymilk manufacture.

4.2. Emulsifying Properties

The ability of proteins to interact with lipids and form stable emulsions is essential to yield a stable food product. Rapeseed protein hydrolysates have higher emulsifying activity (at least 20% greater) and stability than rapeseed protein isolates [94]. Enzymatic hydrolysates of soy protein resulted in an increased emulsification activity [100]. Studies examining protein hydrolysates from different crop sources suggest that the emulsifying capacity of the hydrolysates is related to the degree of hydrolysis, with a low degree of hydrolysis (3%–5%) increasing and a high degree of hydrolysis (~8%) decreasing emulsifying capacity [94,101,102]. Ultrafiltered rapeseed protein hydrolysate was demonstrated to have greater emulsification stability compared to that of whole egg [95] and wheat germ protein hydrolysates had higher emulsification capacity, activity and stability than bovine casein [91]. It is generally accepted that limited hydrolysis improves the emulsification properties of proteins by exposing hydrophobic amino acid residues (which may interact with oil), while the hydrophilic residues interact with water [94]. Similarly, an increase in hydrophilicity as a result of acid modification has been shown to increase the emulsification activity index (EAI) of okara protein isolates [99].

4.3. Foaming

Foaming is of special interest in the food industry as it provides a desirable and unique texture to a range of aerated foods and beverages including ice-cream, bread, cakes, meringues, champagne and beer. It is essential that food foams are stable for consumer acceptability, since consumer perception of quality is influenced by appearance. Wheat germ has been shown to have poor foaming properties [103]. However enzymatic treatment of wheat germ increases foam volume/height but decreases foam stability. The trend of increased foam volume being coupled with decreased foam stability has been reported in previous studies on rice bran protein hydrolysate and acid modification of okara protein hydrolysate [99,104]. The absence of large protein components, which function to stabilise the foam, may contribute to the observed lack of foam stability [93]. In a similar study of wheat germ hydrolysates it was found that foaming capacity was increased at a degree of hydrolysis (DH) of 5%, resulting in a 74% increase in foam volume compared to the control. The foam was also most stable at DH of 5%, with 40% of foam volume sustained after 60 minutes. There was an inverse relationship between DH and foam stability, with stability in the order of DH 5% > DH 10% > DH 15%. Similar to suggestions by Claver *et al.* [93], the stability of the foams was attributed to the presence of larger component proteins and a partial hydrolysis, whereas a higher DH increases the number of polypeptide chains which do not have the ability to stabilise foams [102]. The study of both soy [97] and rapeseed [94] protein hydrolysates produced comparable results. Regarding

the effect of pH on foaming, barley hydrolysates had greater foam stability at basic pH values and very low stability at acidic pH [105].

4.4. Gelation

The ability to manipulate the gel formation properties of a substance is important since gelation is desirable for the bakery and meat industries, but not for foods such as beverages and frozen deserts [106]. As a food ingredient, gelation is one of the most important techno-functional properties of soy protein, however soy protein hydrolysates possess poor gel-forming ability [107]. Transglutaminase (TGase) is a polymeriser shown to be effective in improving gelling of proteins [108], which improved the gelling ability of soy protein hydrolysates [107,109] but the gels formed were inferior to the starting soy protein/isolate in terms of gel strength. It has been suggested that the reduced ability to form gels may be due to lower surface hydrophobicity and short peptide chain length of the hydrolysates [109]. Research has been carried out with a focus on manipulating conditions to give desired gelation properties. For example, it has been shown that sodium chloride (NaCl) concentrations greater than 0.2M can accelerate gelation by sunflower protein hydrolysates but result in a gel of lower strength [110]. Addition of a polysaccharide such as guar gum can enhance the gel strength of canola protein isolates [111] and protein concentration and pH have been identified as important factors influencing gel formation by canola protein isolate coupled with guar gum [112]. Hence, while the gel forming ability of protein hydrolysates from agricultural crops is not as strong as that from proteins, processes are available to improve gelation where necessary.

5. Safety of Protein Hydrolysates

The use of dietary proteins and protein hydrolysates in food products is generally allowed in European countries and has the status of “generally regarded as safe” (GRAS) in the United States of America [113]. In Europe, novel foods are defined as foods and food ingredients that have not been used for human consumption to a significant degree within the European Community before 15 May 1997 [114]. Safety evaluation by external independent experts and approval by competent authorities is necessary before a novel product is allowed on the market. Schaafsma [1] proposed a decision tree that should be used for determining the proposed safety assessment of protein hydrolysates and fractions thereof. Factors for consideration in deciding if a protein hydrolysate should follow procedure for novel foods include documented history of safe use, acceptable food grade hydrolysis process and the effect of intake on amino acid levels [1]. Hydrolysed proteins have a long history of safe use, but it is important to note that the majority of studies in this area look at animal-derived protein hydrolysates in infant feeding practices [114–117]. Some evidence has been produced regarding the sub-chronic toxicity of plant protein hydrolysates. Consumption of potato protein isolates is “well tolerated and without adverse effect” in Wistar rats, with parameters including body weight, body weight gain, mortality and organ weight remaining unchanged [118]. Protein isolates from canola have also been reported to be safe, following a 13 week consumption trial in rats, being practically devoid of natural toxicants and environmental contaminants. The canola protein isolates had no effect on body weight, food consumption, clinical observations, motor activity or ophthalmic examinations [119]. One particular consideration for the safety of protein hydrolysates and bioactive

peptides is their allergenicity. Most allergenic substances are protein-based compounds [120] and peptides in the range of 800–1500 Da are considered non-allergenic [5]. While hydrolysis breaks down proteins into low molecular weight peptides thus lessening the allergenic properties [121], some hydrolysates may retain their parent protein allergenic effects [122]. Therefore, there is a need to further investigate the safety of plant-derived protein hydrolysates and bioactive peptides.

6. Conclusions

Protein hydrolysates derived from agricultural crops have exhibited antioxidant and ACE inhibitory potential, with low molecular weight fractions demonstrating greatest effects. There is substantial evidence which supports the ability of soy protein hydrolysates to reduce CVD risk however hydrolysates obtained from other plant sources require further investigation. Protein hydrolysates show greater potential than intact protein to increase muscle glycogen and muscle protein synthesis and have also demonstrated potential in the treatment of clinical conditions, particularly sunflower hydrolysates which may be used in cases of chronic liver disease. In terms of techno-functional properties, hydrolysates with a low DH have desirable effects on emulsification, foaming and solubility properties. To conclude, protein hydrolysates from agricultural crops have demonstrated favourable bioactive and techno-functional properties that could be exploited for the development of functional foods. For nutraceutical development, clinical trials are necessary to confirm biological activity and safety, in addition to addressing issues such as stability during food processing, organoleptic issues and identifying the fate of plant-derived hydrolysates during passage through the gastrointestinal tract. Furthermore, protein hydrolysates from co-products of the plant processing industry should be investigated for their potential bioactive and techno-functional properties.

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Please note that Appendices (pp. 304-332) are unavailable due to publisher restrictions.

MCCARTHY, A. L., O'CALLAGHAN, Y. C., CONNOLLY, A., PIGGOTT, C. O., FITZGERALD, R. J. & O'BRIEN, N. M. 2012. Phenolic extracts of brewers' spent grain (BSG) as functional ingredients – Assessment of their DNA protective effect against oxidant-induced DNA single strand breaks in U937 cells. *Food Chemistry*, 134, 641-646. <http://dx.doi.org/10.1016/j.foodchem.2012.02.133>

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PAPER

Brewers' spent grain (BSG) protein hydrolysates decrease hydrogen peroxide (H₂O₂)-induced oxidative stress and concanavalin-A (con-A) stimulated IFN- γ production in cell culture

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The present study investigated the bioactivity of protein hydrolysates and fractionated hydrolysates prepared from brewers' spent grain (BSG) using proteases, including Alcalase 2.4L, Flavourzyme and Corolase PP. Hydrolysates were designated K–Y, including fractionated hydrolysates with molecular weight (m.w.) < 3, <5 and >5 kDa. Where computable, IC₅₀ values were lower in U937 (1.38–9.78%) than Jurkat T cells (1.15–13.82%). Hydrolysates L, Q and R and fractionated hydrolysates of U and W (<3, <5, >5 kDa) significantly ($P < 0.01$) protected against hydrogen peroxide (H₂O₂)-induced reduction of superoxide dismutase (SOD) activity. A fractionated hydrolysate of W (<5 kDa) protected against H₂O₂-induced DNA damage, $P < 0.01$. Hydrolysates K, N, P, U, U > 5 kDa, V, V > 5 kDa, W, W > 5 kDa significantly ($P < 0.05$) reduced a concanavalin-A (con-A) stimulated production of interferon- γ (IFN- γ). In conclusion, BSG protein hydrolysates demonstrate bioactivity *in vitro*; lower m.w. hydrolysates (<3, <5 kDa) show greatest antioxidant activity and unfractionated or higher m.w. hydrolysates (>5 kDa) possess anti-inflammatory effects.

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1. Introduction

Brewers' spent grain (BSG) represents 85% of the total solid by-products of the brewing industry, with 8.5 million tons of dry BSG produced on an annual basis globally.¹ This lignocellulosic material is an excellent source of protein and fibre in addition to polyphenolic compounds (hydroxycinnamic acids), vitamins and minerals.^{2,3} Presently, spent grains are sold as livestock feed, particularly for dairy cattle, with a profit in the range of €1–€6 ton⁻¹.⁴ Other potential applications have been suggested; energy and charcoal production, paper manufacture, use in biotechnological processes and as a human food ingredient.²

Meat, eggs, fish, soy and wheat have been highlighted as sources of bioactive peptides,^{5,6} with protein hydrolysates being utilised in many human nutrition products including energy drinks, weight control and sports nutrition products.⁷ In addition to antioxidant and anti-inflammatory potential, protein hydrolysates also possess hypocholesterolemic and anti-hypertensive properties.⁵

Phenolic-rich extracts from BSG have previously demonstrated bioactivity, by protecting against oxidant induced DNA damage.⁸ We previously reported BSG protein isolate and

associated hydrolysates possess selective immunomodulatory potential, significantly reducing Concanavalin-A (con-A) stimulated interferon-gamma (IFN- γ) production.⁹ As a continuation of this research, additional protein hydrolysates were prepared from BSG, with an aim of producing a hydrolysate with enhanced bioactivity. Since molecular weight is one of the main factors influencing biological properties,^{10,11} hydrolysates (produced using Alcalase 2.4L, Corolase PP and Flavourzyme) were fractionated on the basis of differences in molecular mass for bioactivity analysis. It has been reported that membrane separation of protein hydrolysates can result in peptide fractions with enriched bioactivity.¹² Cellular antioxidant activity assays, representing the first line of antioxidant defence in the body, are essential to identify the ability of compounds to intercept oxidative stress and prevent or reduce oxidation.¹³ Furthermore, the comet (single cell gel electrophoresis) assay has been shown to be a successful means of determining the chemoprotective effects of novel functional food ingredients, through antioxidant mechanisms, in cell culture.^{8,14,15} It is hypothesised that fractionation of BSG protein hydrolysates will produce peptide fractions with enriched bioactivity, measured using common assays of bioactivity in cell culture.

The aims of the present study were to assess: firstly, the cytotoxic effects of a second set of BSG protein hydrolysates in human monocytic blood cells (U937) and human leukaemic T cells (Jurkat T); secondly, the cellular antioxidant potential of

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the BSG hydrolysates in the U937 cell line; thirdly, the ability of BSG hydrolysates to protect against H₂O₂-induced DNA damage in U937 cells; and fourthly, the immunomodulatory potential of BSG hydrolysates in con-A stimulated Jurkat T cells.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma-Aldrich Ireland Limited, Co. Wicklow, Ireland, unless otherwise stated. U937 and Jurkat T cells were obtained from the European Collection of Animal Cell Cultures (ECACC).

2.2. Generation of protein rich isolate and protein hydrolysates

The BSG protein rich isolate was obtained by a method previously described.¹⁶ Samples B–T were generated using a BSG protein rich isolate extracted at bench scale whereas samples U–Y were obtained using BSG protein rich isolate extracted at semi-pilot scale. The freeze dried protein rich isolates were then hydrolysed by a range of commercially available enzymes under conditions described in Table 1. The pH was maintained by addition of 0.5 or 2 N 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 hydrolysates were each adjusted to pH 7.0, heated at 95 °C for 10 min to inactivate the enzyme and were then freeze

dried or fractionated using 5 and 3 kDa molecular weight cut-off membranes (Minimate™ Tangential Flow Filtration Capsules, Pall Corporation, New York, USA) and then freeze dried. All samples were prepared at 50 °C (except R, S and T, 60 °C) and pH 7 (except C, R, S and T, pH 9).

2.3. Cell culture

A human monocytic blood cell line (U937 cells) and human leukaemic T cell line (Jurkat T cells) were maintained in antibiotic-free RPMI-1640 medium supplemented with 10% v/v foetal bovine serum (FBS), in a 5% CO₂ atmosphere at 37 °C. Cells were adjusted to a density of 1 × 10⁵ cells per ml for cell proliferation and superoxide dismutase assays and 2 × 10⁵ cells per ml for comet and enzyme-linked immunosorbent assays. Reduced serum media (2.5% FBS) was used for all experiments.

2.4. Cell proliferation

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure cell proliferation. U937 and Jurkat T cells, at a density of 1 × 10⁵ cells per ml, were incubated for 24 h at 37 °C with protein samples at concentrations of 0–10% (v/v) or 0–5% (v/v), respectively, in a 96-well plate. Colour blanks (sample and media only, without cells) were used to allow for possible colour interference by samples. The MTT assay kit (Roche Diagnostics Limited, West Sussex, England) was used to quantify cell proliferation, based on the concept that metabolically active cells convert the yellow tetrazolium MTT salt to a purple formazan derivative. In brief, 10 μl MTT reagent 1 and 100 μl RPMI media were added to each well and incubated for 4 h at 37 °C. Subsequently, 100 μl was then removed and 100 μl MTT reagent 2 added, followed by an overnight incubation at 37 °C, before absorbance at 570 nm was measured spectrophotometrically using a microplate reader (Spectrafluorplus, Tecan). GraphPad Prism 4 was used to calculate the concentration of the samples needed to induce 50% cell death (IC₅₀ value), expressed as % (v/v). A non-toxic concentration of 0.5% (v/v) was chosen for further analyses.

2.5. Superoxide dismutase (SOD) assay

Using a Calbiochem SOD Assay Kit (Merck Chemicals Ltd, Nottingham, UK), SOD activity was measured. U937 cells (5 ml, 1 × 10⁵ cells per ml) were incubated for 24 h with 0.5% (v/v) test samples at 37 °C, followed by a 60 min exposure to 100 μM H₂O₂ at 37 °C. SOD activity is expressed relative to the protein content of the samples, measured using the Bicinchoninic Acid Protein (BCA) assay (previously described¹⁷), as SOD units per mg protein cell homogenate (U mg⁻¹ protein). The amount of SOD enzyme needed to dismutate 50% of the superoxide radical (O₂⁻) is equivalent to 1 SOD unit.

2.6. Single cell gel electrophoresis (SCGE) – comet assay

U937 cells (2 ml, 1 × 10⁵ cells per ml) were incubated for 24 h at 37 °C with 0.5% (v/v) test samples and subsequently exposed to 50 μM H₂O₂ for 30 min. Using the fluorescein diacetate ethidium bromide (FDA/EtBr) assay, cell viability was checked

Table 1 Summary of methods used to prepare brewers' spent grain (BSG) protein hydrolysates

Sample	Description	Enzyme/water	E/S ratio (w/w or v/w) %
B	Control for K–Q	Water	—
C	Control for R–T	Water	—
K	BSG protein hydrolysate	Protamex	1
L	BSG protein hydrolysate	Prolyve	1
M	BSG protein hydrolysate	Protex 6L	1
N	BSG protein hydrolysate	Promod 144 MG	1
O	BSG protein hydrolysate	Promod 24P	1
P	BSG protein hydrolysate	Trypsin 250	1
Q	BSG protein hydrolysate	Corolase L10	1
R	BSG protein hydrolysate	Alcalase 2.4L	2.5
S	5 kDa retentate	Alcalase 2.4L	2.5
T	5 kDa permeate	Alcalase 2.4L	2.5
U	BSG protein hydrolysate	Alcalase 2.4L	1
U < 3	3 kDa permeate	Alcalase 2.4L	1
U < 5	5 kDa permeate	Alcalase 2.4L	1
U > 5	5 kDa retentate	Alcalase 2.4L	1
V	BSG protein hydrolysate	Corolase PP	1
V < 3	3 kDa permeate	Corolase PP	1
V < 5	5 kDa permeate	Corolase PP	1
V > 5	5 kDa retentate	Corolase PP	1
W	BSG protein hydrolysate	Flavourzyme	1
W < 3	3 kDa permeate	Flavourzyme	1
W < 5	5 kDa permeate	Flavourzyme	1
W > 5	5 kDa retentate	Flavourzyme	1
X	Unhydrolysed BSG protein	—	—
Y	Control for U–W	Water	—

and was consistently >90%. The comet assay, as previously described,⁸ was used to determine the effect of the extracts on oxidant-induced DNA damage. Briefly, cells were harvested and embedded on microscope slides (prepared with normal gelling agarose (NGA)) using low melting point agarose (LMP). Cells were placed in lysis solution for 1 h at 4 °C, followed by electrophoresis at 300 mA, 21 V for 25 min. Slides were neutralised by using Tris (0.4 M) and stained with EtBr (20 µg ml⁻¹). Cells were visualised using a fluorescence microscope (Optiphot-2, Nikon) and Komet 5.5 image analysis software. For each slide, 50 cells were scored and DNA damage expressed as percentage tail DNA.

2.7. Enzyme-linked immunosorbent assay (ELISA) – interferon-γ production

Jurkat T cells were treated with concanavalin-A (con-A, 50 µg ml⁻¹) and incubated for 24 h at 37 °C with 0.5% (v/v) test samples. eBioscience ELISA kit (Human IFN-γ ELISA Ready-Set-Go) was used to measure the production of interferon-γ (IFN-γ). Absorbance was read at 450 nm using a microplate reader (ThermoScientific Varioskan Flash).

2.8. Statistical analysis

All data points represent the mean ± standard error (s.e.) of at least 3 independent experiments. Statistical analysis was carried out using GraphPad Prism 4.00 (GraphPad Software, California,

USA); analysis was by one-way analysis of variance (ANOVA) followed by Dunnett's test (or Tukey's multiple comparison test, where stated).

3. Results

For hydrolysate samples K–Q and R–T the control protein samples are labelled B and C, respectively, as detailed in Table 1. These control samples, B and C, were previously studied in our earlier publication.⁹ The control for samples U,V, W and associated fractionated hydrolysates is sample Y.

3.1. Cell proliferation

The effect of test samples on cell proliferation in both U937 and Jurkat T cells was measured using the MTT assay. Concentrations between 0 and 10% (v/v) were analysed in the U937 cell line, as shown in Table 2 and Fig. 1. Sample Y was the most cytotoxic, with 0.5% (v/v) significantly ($P < 0.05$) decreasing cell proliferation, while M was the least toxic sample in the U937 cell line. The concentration of each sample needed to induce 50% cell death (IC₅₀ value) was calculated. In the U937 cell line this was found to range from 1.38% (v/v) to non-determinable (n/d). Therefore, for further analyses using U937 cells, a non-toxic concentration of 0.5% (v/v) was chosen.

The maximum concentration tested was 5% (v/v) for the MTT assay in the Jurkat T cell line, as results in the U937 cell line suggested that concentrations in excess of 5% (v/v) to be

Table 2 Effect of BSG protein hydrolysates (0–10% (v/v)) on cell proliferation in the U937 cell line

Sample	Cell proliferation (% of control) ^a										IC ₅₀ ^b (% (v/v))
	0%	0.5%	1%	2%	2.5%	4%	5%	6%	8%	10%	
K	100.00	95.73	89.53	95.15	84.48	80.59	95.85	62.87	31.14*	29.32*	6.02
L	100.00	96.78	94.24	111.87	99.52	74.98	133.34	73.18	37.20*	40.89*	6.01
M	100.00	82.93	113.92	104.41	104.80	105.21	114.64	94.67	64.59	64.75	n/d
N	100.00	96.22	88.21	100.04	100.03	125.80	89.08	75.17	49.97	28.51*	8.13
O	100.00	83.97	89.82	89.69	105.13	86.77	107.16	65.09	54.07	31.39*	5.98
P	100.00	93.94	93.63	87.09	112.62	74.10	103.54	88.07	64.07	25.67*	7.84
Q	100.00	87.43	92.08	70.36	103.53	78.17	87.01	73.79*	52.25*	19.75*	9.07
R	100.00	88.23	98.34	81.01	88.22*	30.72*	17.92*	13.84*	55.21	37.16*	2.90
S	100.00	88.42	81.84	68.11	50.58*	20.48*	31.80	29.04*	55.39	74.18	2.01
T	100.00	82.11	58.56*	43.43*	49.05*	63.81*	43.25*	35.99*	50.55*	33.69*	5.84
U	100.00	80.01	88.26	40.77*	29.13*	44.77*	46.09*	30.21*	44.36*	26.86*	1.94
U < 3	100.00	94.42	109.62	99.04	89.10	62.27	88.79	81.67	54.90*	25.29*	9.78
U < 5	100.00	76.21	103.72	95.68	84.25	86.47	83.97	72.53	53.24	30.04*	9.34
U > 5	100.00	62.93	89.92	76.59	50.91	39.97*	22.64*	18.17*	9.12*	-5.14*	4.27
V	100.00	45.00	114.60	82.22	84.36	25.39*	26.63*	23.92*	19.22*	15.88*	3.53
V < 3	100.00	69.94	66.46	77.75	84.42	68.63	63.76	49.07*	34.61*	23.20*	6.33
V < 5	100.00	60.32*	71.44	73.73	83.21	65.12*	71.20	57.27*	38.03*	25.47*	7.19
V > 5	100.00	57.32	94.43	60.37	38.48*	31.00*	45.52*	32.53*	5.63*	12.12*	4.64
W	100.00	79.36	80.68	23.74*	23.58*	17.90*	24.60*	19.20*	14.47*	10.69*	1.57
W < 3	100.00	74.87	78.81	83.82	71.45	61.35	31.48*	13.66*	16.27*	3.76*	4.59
W < 5	100.00	76.07	77.60	57.24*	54.91*	55.93*	43.07*	34.37*	24.54*	8.04*	5.87
W > 5	100.00	66.23	77.95	68.44	20.71*	12.48*	14.66*	-1.22*	16.41*	-2.31*	2.32
X	100.00	68.15	38.82*	35.89*	48.32*	34.51*	29.27*	31.82*	38.03*	24.68*	3.56
Y	100.00	72.63*	58.92*	38.00*	17.74*	17.68*	17.85*	12.62*	25.05*	-8.22*	1.38

^a Values represent the mean ± s.e. of 3 independent experiments. ^b Values relate to the concentration of sample added to the cells, expressed as % (v/v) and were computed using GraphPad Prism 4.00. * Denotes statistically significant ($P < 0.05$) difference in cell proliferation relative to untreated control U937 cells. Statistical analysis by ANOVA followed by Dunnett's test.

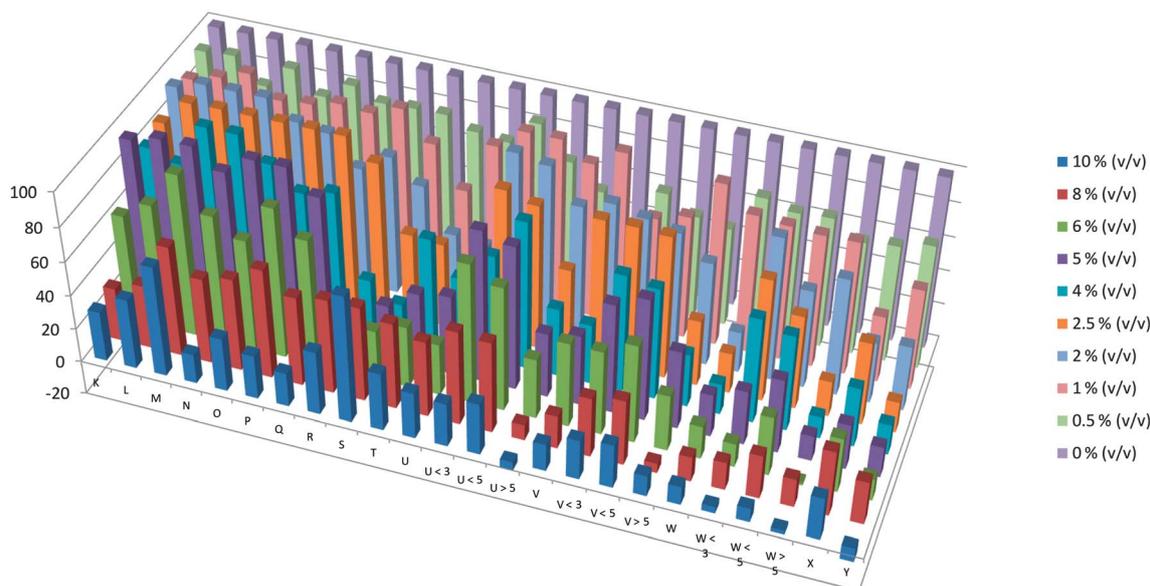


Fig. 1 Effect of BSG protein hydrolysates (0–10% v/v) on cell proliferation in the U937 cell line. Values represent the mean of 3 independent experiments.

unsuitable for experimentation, having significant ($P < 0.05$) effects on cell proliferation. Results of the MTT assay in the Jurkat T cell line are shown in Table 3 and Fig. 2. The samples tested were less cytotoxic in Jurkat T cells than in U937 cells, with the IC_{50} values of 10 samples not determinable. The IC_{50}

values of the remaining samples were in the range of 1.15–13.82% (v/v). Similar to the effects in U937 cells, sample Y was the most cytotoxic. Therefore, a non-toxic concentration of 0.5% (v/v) was chosen for immunomodulatory analysis of the samples in the Jurkat T cell line.

Table 3 Effect of BSG protein hydrolysates (0–5% (v/v)) on cell proliferation in the Jurkat T cell line

Sample	Cell proliferation (% of control) ^a							IC_{50}^b (% v/v)
	0%	0.5%	1%	2%	2.5%	4%	5%	
K	100.00	78.32	75.55	65.93	78.85	52.44	59.97	n/d
L	100.00	86.38	80.24	75.17	86.51	53.79*	65.38	n/d
M	100.00	78.59	84.88	83.78	89.78	52.37*	76.53	n/d
N	100.00	83.26	87.10	83.96	97.01	65.28	72.88	n/d
O	100.00	94.31	99.21	84.72	95.50	59.75	83.06	n/d
P	100.00	88.38	98.29	81.82	100.43	73.10	67.78	12.56
Q	100.00	89.00	94.09	89.69	109.40	102.77	114.42	n/d
R	100.00	80.58	85.38	73.63	49.78	49.08	66.36	n/d
S	100.00	80.23	79.59	70.80	60.99	17.81*	15.32*	5.70
T	100.00	89.65	85.04	93.08	69.79*	81.69	80.57	6.59
U	100.00	71.03	69.39	47.85*	16.20*	38.46*	35.80*	1.99
U < 3	100.00	58.47*	57.22*	64.17	66.51	71.60	69.85	2.08
U < 5	100.00	79.23	86.17	72.87	73.00	81.38	65.61	11.44
U > 5	100.00	66.13*	67.39	44.67*	18.13*	21.19*	35.40*	2.00
V	100.00	71.63*	74.82*	68.00*	51.79*	29.34*	33.76*	2.48
V < 3	100.00	70.39*	66.20*	62.59*	62.29*	64.55*	51.23*	7.14
V < 5	100.00	75.49	70.92	64.85*	78.06	70.53	75.72	13.82
V > 5	100.00	60.90*	63.56*	9.52*	12.90*	13.92*	12.87*	3.25
W	100.00	63.54	58.09	46.62*	27.75*	27.90*	27.42*	2.02
W < 3	100.00	67.24	59.24*	65.03	60.78*	68.24	64.55	n/d
W < 5	100.00	78.41	67.05	71.12	66.13	57.69	76.92	n/d
W > 5	100.00	55.89	72.16	50.98	39.98	45.22	39.75	n/d
X	100.00	63.36*	63.65*	19.29*	15.56*	48.11*	73.99	4.12
Y	100.00	70.12	68.48	27.16*	28.60*	24.14*	40.79*	1.15

^a Values represent the mean \pm s.e. of 3 independent experiments. ^b Values relate to the concentration of sample added to the cells, expressed as % (v/v) and were computed using GraphPad Prism 4.00. * Denotes statistically significant ($P < 0.05$) difference in cell proliferation relative to untreated control U937 cells. Statistical analysis by ANOVA followed by Dunnett's test.

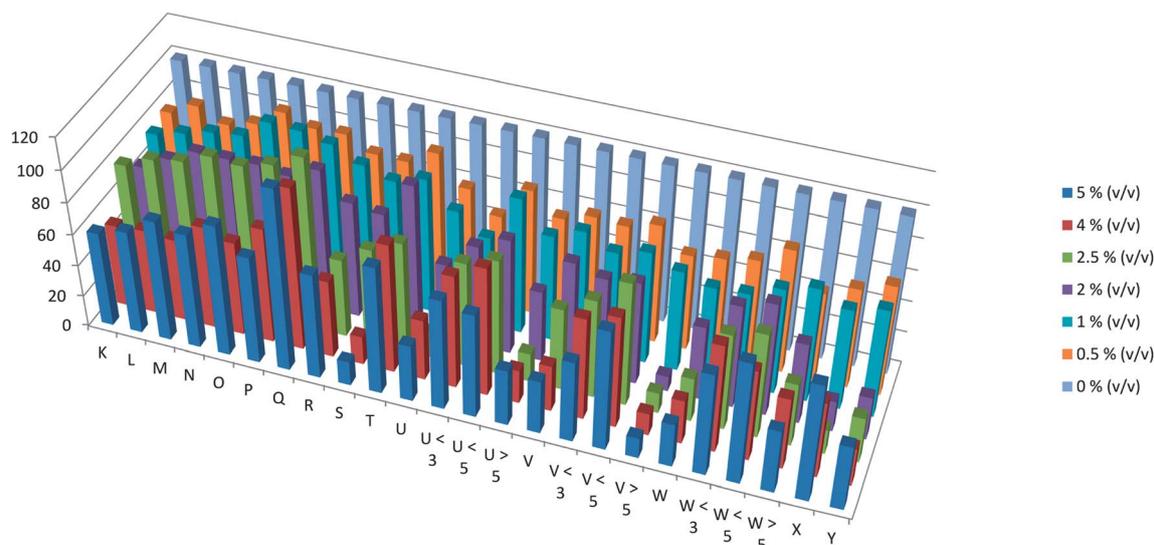


Fig. 2 Effect of BSG protein hydrolysates (0–5% v/v) on cell proliferation in the Jurkat T cell line. Values represent the mean of 3 independent experiments.

Hydrolysates U, V and W and fractionated hydrolysates of U and V had significantly ($P < 0.01$) higher IC_{50} values than that of control sample Y, in both cell lines; indicating that fractionated hydrolysates, particularly with molecular weight (m.w.) < 3 and < 5 kDa, are less cytotoxic than the parent hydrolysate samples.

3.2. Antioxidant activity

The antioxidant potential of the samples was measured by their ability to protect against H_2O_2 -induced damage in U937 cells using two assays; SOD and comet assays. Exposure of U937 cells to $100 \mu M H_2O_2$ for 60 min resulted in a significant ($P < 0.01$) decrease in SOD activity to 57.24% (Table 4). Samples L, Q and R significantly ($P < 0.01$) protected against this oxidant-induced reduction in SOD activity. A number of the fractionated hydrolysates also provided significant ($P < 0.01$) protection against H_2O_2 induced decrease in SOD activity; U < 3 kDa, U < 5 kDa, U > 5 kDa, V > 5 kDa, W < 3 kDa, W < 5 kDa, W > 5 kDa.

Protection against DNA damage induced by $50 \mu M H_2O_2$ for 30 min was measured using the comet assay. Examples of the cell-images scored using image analysis software are shown in Fig. 3. H_2O_2 significantly ($P < 0.01$) increased the percentage tail DNA to 41.79% (Fig. 4). The fractionated hydrolysate W < 5 kDa significantly ($P < 0.01$) protected against H_2O_2 -induced DNA damage, while other samples did not exert significant antioxidant effects (Fig. 4). However, a general trend was observed indicating that the fractionated hydrolysates reduced the % tail DNA to a greater extent than their corresponding unfractionated samples (Fig. 4).

3.3. Immunomodulatory potential

ELISA was used to measure the ability of the BSG protein samples to protect against the con-A stimulated production of IFN- γ in Jurkat T cells; results are shown in Table 5. Sample K showed most anti-inflammatory potential, significantly ($P < 0.05$) reducing IFN- γ production to 53.28%. Other samples also significantly ($P < 0.05$) reduced IFN- γ levels by a minimum of

12% (W) and maximum of 28% (P). Unfractionated hydrolysates U, V and W significantly ($P < 0.001$) reduced IFN- γ production to a greater extent than the corresponding fractionated samples.

Table 4 Ability of BSG protein hydrolysates (0.5% (v/v)) to protect against H_2O_2 -induced oxidative stress in the U937 cell line, measured by superoxide dismutase (SOD) activity^a

Sample	SOD activity (U mg^{-1} protein)	SOD activity (% of control)
Control	0.36 ± 0.01	100 ± 0.00
H_2O_2 control	$0.18 \pm 0.00^*$	$57.24 \pm 0.70^*$
K	0.28 ± 0.06	91.73 ± 16.82
L	$0.57 \pm 0.10^\#$	$140.47 \pm 9.29^\#$
M	0.32 ± 0.03	94.75 ± 8.22
N	0.28 ± 0.02	82.21 ± 13.00
O	0.30 ± 0.04	87.62 ± 10.60
P	0.34 ± 0.10	94.51 ± 15.53
Q	0.35 ± 0.06	$109.24 \pm 25.03^\#$
R	0.39 ± 0.02	$118.93 \pm 13.65^\#$
S	0.32 ± 0.06	91.26 ± 10.44
T	0.30 ± 0.03	88.16 ± 10.43
U	0.24 ± 0.12	67.18 ± 5.38
U < 3	0.36 ± 0.20	$101.87 \pm 4.86^\#$
U < 5	0.40 ± 0.12	$111.51 \pm 3.31^\#$
U > 5	0.29 ± 0.09	$80.53 \pm 4.33^\#$
V	0.22 ± 0.05	62.79 ± 3.53
V < 3	0.23 ± 0.20	65.94 ± 7.12
V < 5	0.24 ± 0.09	67.22 ± 4.09
V > 5	0.33 ± 0.10	$91.99 \pm 2.81^\#$
W	0.25 ± 0.06	70.78 ± 4.24
W < 3	0.44 ± 0.14	$124.39 \pm 2.89^\#$
W < 5	0.31 ± 0.22	$87.72 \pm 3.54^\#$
W > 5	0.39 ± 0.04	$108.44 \pm 1.81^\#$
X	0.21 ± 0.03	58.29 ± 1.95
Y	0.17 ± 0.03	48.24 ± 3.34

^a Data represents the mean \pm s.e. of 4 independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * Denotes statistically significant difference in SOD activity between control and H_2O_2 control ($P < 0.05$). # Denotes statistically significant difference in SOD activity between H_2O_2 control and protein hydrolysate ($P < 0.01$).

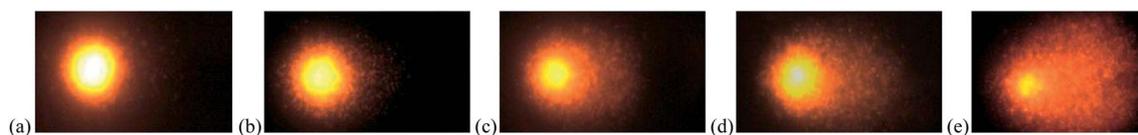


Fig. 3 Cellular DNA damage induced by H_2O_2 , as measured by the comet (single cell gel electrophoresis) assay in U937 cells and visualised using Komet 5.5 image analysis software. (a) Control, untreated U937 cell (b–e) U937 cells with increasing degrees of DNA damage.

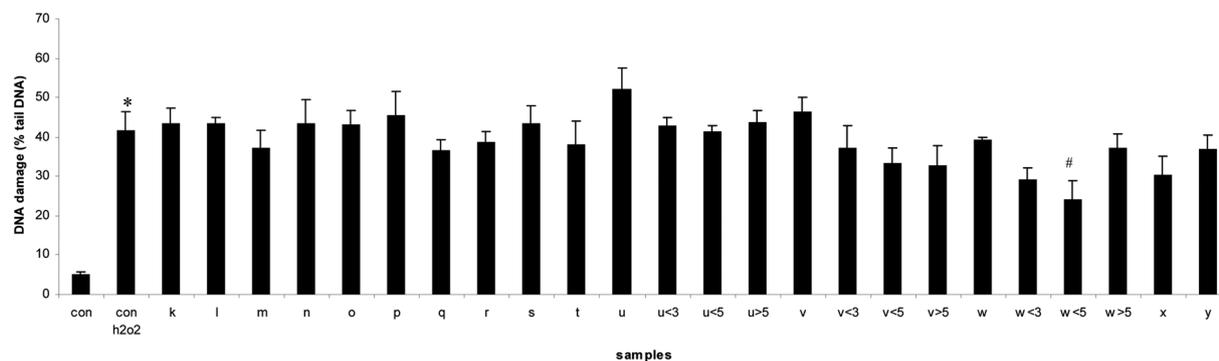


Fig. 4 The ability of protein hydrolysates from brewers' spent grain (BSG) to protect against H_2O_2 -induced DNA damage in the U937 cell line, measured by the comet assay. Data represents the mean \pm s.e. of 3 independent experiments. Statistical analysis by ANOVA followed by Dunnett's test. * Denotes statistically significant difference in DNA damage (% tail DNA) between control and H_2O_2 control ($P < 0.01$). # Denotes statistically significant difference in DNA damage (% tail DNA) between H_2O_2 control and BSG protein hydrolysate ($P < 0.01$).

4. Discussion

BSG is a low-value co-product of the brewing industry, with a composition of 20% protein and 70% fibre.² Phenolic extracts of BSG have previously demonstrated antioxidant capacity⁸ and BSG protein hydrolysates have been shown to possess selective anti-inflammatory effects.⁹ The bioactivity of further protein hydrolysates, including fractionated hydrolysates, was the focus of the present study.

The MTT cell proliferation assay was used to determine the cytotoxicity of the samples in both the U937 and Jurkat T cell lines and to indicate an appropriate concentration for further analyses. In agreement with previously reported data for BSG hydrolysates, the present set of hydrolysates were more cytotoxic in U937 than in Jurkat T cells.⁹

Superoxide dismutases (SODs) are a group of three metalloenzymes, copper/zinc (Cu/Zn), manganese (Mn) and iron (Fe) SOD, involved in catalysing the dismutation of the superoxide anion (O_2^-) to molecular oxygen (O_2) and H_2O_2 in humans. The assay kit utilised in this study measures all three SODs generated by xanthine oxidase and hypoxanthine. Antioxidant enzymes such as SOD and catalase (CAT) function in minimising oxidative stress. However, the oxidant interception and prevention process is not always entirely effective and products of damage can be continually formed at low levels, leading ultimately to protein and DNA damage (including single-strand breaks).¹³ The comet assay measures the ability of a sample to protect against oxidant-induced single strand breaks in DNA (DNA damage) *in vitro*. To determine the antioxidant effects of BSG protein hydrolysates, two different antioxidant assays were used – SOD and comet assays, to measure both interception and

repair of oxidative stress. A number of protein hydrolysates demonstrated the ability to protect against the H_2O_2 -induced reduction in SOD activity in the U937 cell line (Table 4). Interestingly, the fractionated hydrolysates of U and W had significant antioxidant effects, by the SOD assay ($P < 0.01$), while the parent hydrolysates did not show significant effects. This suggests that the lowest m.w. hydrolysate fractions (<3 and <5 kDa) had the greatest antioxidant capability of hydrolysates U–Y. Sample W < 5 kDa was the only BSG protein fraction to show significant ($P < 0.01$) ability to repair DNA, protecting against oxidant-induced DNA damage as assessed by the comet assay. This hydrolysate fraction reduced DNA damage from 41.79 to 21.02% tail DNA and also increased SOD activity from 57.24 to 87.72%. The greater antioxidant potential of the lower m.w. (<5 kDa) hydrolysates supports previous findings in the literature for quinoa seed protein hydrolysates, <5 kDa¹⁸ and rapeseed protein isolates, <1 kDa.¹⁹

Hydrogen peroxide (H_2O_2) induces oxidative stress in an iron-dependent manner; producing hydroxyl radicals (OH) *via* the Fenton reaction. The ability of BSG protein hydrolysates to protect against H_2O_2 -induced oxidative stress suggests their ability to scavenge ferrous iron (Fe^{2+}), thus making it unavailable for reaction. The ability of proteins to inhibit oxidation by binding iron has been discussed elsewhere.²⁰ In accordance with previous reports,²¹ the main amino acids present in the BSG hydrolysates have been found to be in the order of glutamine > proline > leucine.¹⁶ Glutamic acid is the amino acid most highly correlated with antioxidant activity of Spanish honeys.²² Similarly, pea seed protein hydrolysates with highest antioxidant activity have highest glutamic and aspartic acid contents.²³ Hence, the presence of glutamine in the BSG protein

Table 5 The effect of BSG protein hydrolysates (0.5% (v/v)) on IFN- γ production in concanavalin-A (con-A) stimulated Jurkat T cells^a

BSG hydrolysate	IFN- γ production (% of control)
Control	100.00 \pm 0.00
K	53.28 \pm 17.17*
L	74.28 \pm 4.97
M	76.30 \pm 6.29
N	72.68 \pm 6.74*
O	75.88 \pm 3.84
P	71.16 \pm 8.94*
Q	73.39 \pm 12.66
R	60.67 \pm 13.58
S	58.32 \pm 13.46
T	72.65 \pm 13.44
U	77.70 \pm 2.34*
U < 3	98.19 \pm 2.81#
U < 5	102.56 \pm 3.78#
U > 5	81.95 \pm 2.28*
V	86.86 \pm 1.41*
V < 3	99.55 \pm 2.06#
V < 5	105.57 \pm 3.61#
V > 5	81.32 \pm 2.05*
W	87.27 \pm 1.47*
W < 3	113.47 \pm 4.10#
W < 5	95.15 \pm 3.20
W > 5	78.57 \pm 1.56*
X	83.12 \pm 1.68*
Y	80.88 \pm 4.95*

^a Data represents the mean \pm s.e. of 3 independent experiments. Statistical analysis by ANOVA followed by Tukey's multiple comparison test. * Denotes statistically significant reduction in IFN- γ production, relative to con-A treated Jurkat T cells control ($P < 0.05$). # Denotes statistically significant difference in IFN- γ production, relative to unfractionated parent compound ($P < 0.001$).

hydrolysates may be, at least partially, responsible for their antioxidant activity.

The antioxidant activity of food proteins can be attributable to a number of characteristics. Peptides with 5–16 amino acid residues, particularly tyrosine, tryptophan, methionine, lysine, cysteine and histidine are considered to be antioxidant peptides.²⁴ Peptide linkage, configuration and concentration, position of amino acids in the peptide sequence, molecular weight and degree of hydrolysis can all contribute to antioxidant activity and the integration of these effects determines overall antioxidant potential.²⁴ Furthermore, non-purified food peptides (protein hydrolysates) have higher absorption and antioxidant activity than purified peptides.²⁴

The selective immunomodulatory potential of BSG protein hydrolysates was highlighted in a previously published study.⁹ These hydrolysates demonstrated the ability to reduce the con-A stimulated production of IFN- γ in the Jurkat T cell, no effect was seen on interleukins 2 (IL-2), 4 (IL-4) or 10 (IL-10).⁹ On the basis of these results, it was decided for the purposes of the present study that potential modulation of IFN- γ production would be measured in con-A stimulated Jurkat T cells. Hydrolysates K, N, P, U, V and W showed significant ($P < 0.05$) anti-inflammatory

activity. Interestingly, hydrolysates U, V and W significantly ($P < 0.01$) reduced IFN- γ production to a greater extent than the low m.w. hydrolysate fractions <3 and <5 kDa (except W < 5 kDa). This suggests that unfractionated hydrolysates or fractions with m.w. > 5 kDa possess greatest anti-inflammatory potential. This is further supported by the finding that of the fractionated hydrolysates, only fractions >5 kDa significantly ($P < 0.05$) reduced IFN- γ production. IFN- γ is a type 2 pro-inflammatory cytokine²⁵ that plays a crucial role in the immune system by secreting chemokines, activating immune cells, regulating antigen presentation and controlling T-lymphocyte adaptive immune responses.²⁶ Hence, the ability to inhibit production of IFN- γ suggests anti-inflammatory activity. Events such as tissue injury, microbial invasion and oxidative stress can induce acute inflammation²⁷ mediated by a number of mediators including cytokines and nitric oxide.²⁸ A link between antioxidant and anti-inflammatory activities has previously been reported for whey protein hydrolysates,^{29,30} and BSG hydrolysates demonstrating anti-inflammatory potential in the present study also significantly increased SOD activity.

In T lymphocytes, it is thought that con-A stimulates cytokine production by activating mitogen-activated protein kinase (MAPK) and nuclear factor of activated T cell (NFAT) *via* a T-cell receptor.³¹ Compounds with the ability to suppress cytokine production induced by con-A in T lymphocytes can act by inhibiting protein kinase C (PKC) or calcineurin (CN) activity.³¹ Hence, BSG protein hydrolysates may be exerting anti-inflammatory effects by these mechanisms.

Chronic inflammation may occur following acute inflammation and result in persistent infection and autoimmune diseases.²⁷ Such inflammatory and autoimmune diseases include rheumatoid arthritis, inflammatory bowel disease and psoriasis.³² It has been postulated that IFN- γ plays a role in chronic inflammation by acting as a macrophage activating factor (MAF) and a migration inhibition factor (MIF), both activating macrophages and retaining them at the inflammatory site.²⁸ Therefore, the discovery of novel anti-inflammatory compounds, particularly with the ability to reduce production of IFN- γ , has implications for human health.

The hydrolysates for the present study were produced by a number of enzymes including Protamex, Alcalase 2.4L, and Flavourzyme (Table 1). Alcalase is a *Bacillus licheniformis* preparation containing subtilisin and glutamyl endopeptidase activities.³³ Flavourzyme is from *Aspergillus oryzae* and contains proteinase and exopeptidase activities.³⁴ BSG protein hydrolysates M and O prepared with Protex 6L and Promod 24P, respectively, did not show antioxidant or anti-inflammatory potential. Contrastingly, hydrolysates prepared with Alcalase 2.4L, CorolasePP, and Flavourzyme at 50 °C, pH 7 and at an E/S of 1% (v/v) demonstrated greatest bioactivity. It has previously been reported that there is great variation in the performance of commercial enzymes, with a wide range of protease variants and specificities available.^{35,36} Alcalase has been repeatedly documented as being the most effective in terms of degree of hydrolysis^{36–38} and bioactivity^{38,39} of resulting hydrolysates. Flavourzyme is also a favourable protease for the production of bioactive protein hydrolysates.^{40,41}

By comparing the results of the current study to those of the previous study of BSG protein hydrolysates,⁹ it is evident that the present set of protein hydrolysates are more bioactive. While the ability to reduce IFN- γ production is demonstrated in the results of both studies, antioxidant activity of BSG protein hydrolysates is only evident in the present study. This may be attributable to differences arising during scaling-up of the initial protein extraction procedure to semi-pilot scale prior to hydrolysis, coupled with the enzymatic conditions (*i.e.* E/S ratio 1%, pH 7, temperature of 50 °C) and the fractionation of hydrolysates.

5. Conclusion

Results of the present study suggest BSG protein hydrolysates possess antioxidant and anti-inflammatory potential. Hydrolysates with lower molecular weight (<3 and <5 kDa) have greatest antioxidant activity, whereas unfractionated hydrolysates or hydrolysates with m.w. > 5 kDa appear to have greater anti-inflammatory effects. These bioactive hydrolysates demonstrate potential for incorporation into functional foods, for the management of oxidation and inflammation associated diseases.

Conflict of interest

The authors declare no conflict of interest.

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