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Investigating the antioxidant, immunomodulatory and anti-obesogenic propertiesof novel BSG-derived extracts and their potential as ingredients in functional foods for older adults

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Declaration

I certify that this thesis which I now submit for examination for the award of Master by Research (MSc) is entirely my own work and has not been taken from the work of others and to the extent that such work has been cited and acknowledged within the text of my work.

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Signed: Suzanne O'Brien Date: 02/09/2020

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Abstract

Brewer's Spent Grain (BSG) represents a substantial waste product of the brewing industry, with 39 million tonnes generated globally every year. To date the primary use of BSG is as animal feed but there is significant interest in identifying alternative uses for this agro-industry by-product. BSG is rich in protein and fibre and is known to contain health-promoting bioactive compounds, thus one application would be in the area of functional foods. People are living longer and as the older adult population continues to increase globally so too is the prevalence of age-related chronic conditions. Diet plays an essential role in healthy ageing and there is currently an increased demand for more convenient, affordable and healthy food options for the elderly cohort.

This research aims to help reduce the environmental burden of brewery waste and add to the global demand for innovative functional food products for older adults, by investigating the bioactivity of BSG. Novel BSG fractions were generated, digested with a simulated gastrointestinal *in vitro* digestion (SGID) system and then screened for a range of bioactivities. The potential antioxidant, immunomodulatory and anti-obesogenic properties of the digestates were investigated using cellular and non-cellular assays. BSG-derived fractions that had significantly improved ($p<0.05$) antioxidant properties were identified. Anti-obesity investigations revealed that all BSG fractions significantly reduced ($p<0.05$) adipocyte differentiation *in vitro*, but none of the fractions inhibited lipase activity. The anti-obesity properties of BSG have not been previously investigated. Data from this study provides additional knowledge on the bioactive properties of BSG and gives additional value to brewing co-products. The fractions generated in this study will support the use of BSG and BSG-derived fractions as ingredients in the design of functional foods.

Abbreviations

6-OHDA: 6-hydroxydopamine

AAPH: 2,2′-azobis(2-amidinopropane) Dihydrochloride

ACE: Angiotensin-Converting Enzyme

ADA: Adenosine Deaminase,

ADP: Adenosine Diphosphate

Alc: Alcalase

ALP: Alkaline Phosphatase

ALT: Alanine Transaminase

AMPK: 5' Adenosine Monophosphate-Activated Protein Kinase

AST: Aspartate Transaminase

AX: Arabinoxylan

BMI: Body Mass Index

$CaCl_2$: Calcium Chloride

$CaCO_3$: Calcium Carbonate

CaMKKβ: Ca(2+)/Calmodulin-Dependent Protein Kinase Kinase-β

CAT: Catalase

CH_3COONa: Sodium Acetate

CIS: Cisplatin

DEX: Dexamethasone

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl Sulfoxide

DPPH: 2, 2-diphenyl-1-picrylhydrazyl

eNOS: Endothelial Nitric Oxide Synthase

E-NTPDase: Ectonucleoside Triphosphate Diphosphohydrolase

$FeCl_3$: Iron(III) Chloride / Ferric Chloride

$FeSO_4.7H_2O$: Iron (II) Sulfate Heptahydrate ACS reagent ≥ 99.0%

Fla: Flavourzyme

FOXO3: Forkhead Box O3

FRAP: Ferric Reducing Antioxidant Power

GAE: Gallic Acid Equivalents

GGT: γ-Glutamyl Transferase / Gamma-Glutamyl Transferase

GPx: Glutathione Peroxidase

GSH: Glutathione

HCL: Hydrochloric Acid

HDL: High-Density Lipoprotein

IBMX: 3-Isobutyl-1-Methylxanthine

IFN-γ: Interferon Gamma

IL-*1β*: Interleukin-1 β / Interleukin 1 beta

IL-2: Interleukin-2

IL-6: Interleukin 6

IOM: Institute of Medicine

LPS: Lipopolysaccharide

MAPKs: Mitogen-activated protein kinases

MetOH: Methanol

$MgCl_2$: Magnesium Chloride

mRNA: Messenger Ribonucleic Acid

NaCl: Sodium Chloride

Na_2CO_3: Sodium Carbonate

Na_2HPO_4: Sodium Hydrogen Phosphate

NaOH: Sodium Hydroxide

NBCS: New Born Calf Serum

NO: Nitric Oxide

NPB: 4-Nitrophenyl Butyrate / p-Nitrophenyl Butyrate,

PBS: Phosphate Buffered Saline

PLC: Phospholipase C

PLC-PKC: Phospholipase C - Protein Kinase C

PPARα: Peroxisome Proliferator-Activated Receptor Alpha

PPAR-γ: Proliferator-Activated Receptor Gamma

RDA: Recommended Dietary Allowance

RDI: Recommended Dietary Intake

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

SOD: Superoxide Dismutase

SREBP1: Sterol Regulatory Element-Binding Protein 1

SYR: Syringaresinol

TG: Triglyceride

TILDA: The Irish Longitudinal Study on Ageing

TPC: Total Phenolic Content

Trolox: (±)-6-Hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid

WST-1: (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt)/Water-soluble tetrazoliuM -1

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Chapter 1

Literature Review - Brewer's spent grain-derived bioactive ingredients; their role in elderly nutrition and potential for functional food development

1.0 Introduction

An older adult is defined as a person aged ≥ 65 years (Singh & Bajorek, 2014). In 2019, a total of 703 million elderly people were accounted for in the world (United Nations, 2019). People are living longer and by 2050, the global population of older adults is set to further increase (Figure 1.1 and Figure 1.2) and reach two billion (Shlisky et al., 2017). In 2017 Japan, Italy, Germany, Portugal, Finland, Bulgaria, Croatia, Greece, Slovenia and Latvia were the ten countries reported to have the greatest share of a 60+ population (>25%), however in 2050, 5 of the 10 most aged countries are expected to be in Europe (United Nations, 2017). A demographic projection for Ireland indicates that the 65+ population is estimated to reach 637,567 in 2051, with female life expectancy to rise to 88 years and male life expectancy to rise to 85 years (Mealy & Sorensen, 2020). By 2030 the world centenarian population is estimated to more than double and by 2050 reach 3.4 million (Bloom et al., 2016a). Japan is populated with the largest percentage (30%) of people aged 60+ years and will increase to 44% by 2050 (Bloom et al., 2016a).

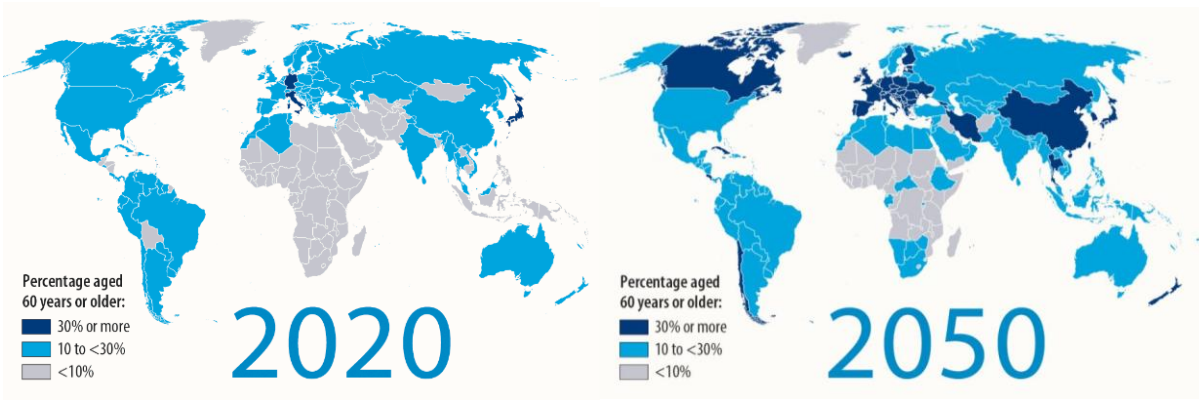

Figure 1.1: Global population aged ≥ 60 years, 2020 vs. 2050 (World Health Organization, 2015)

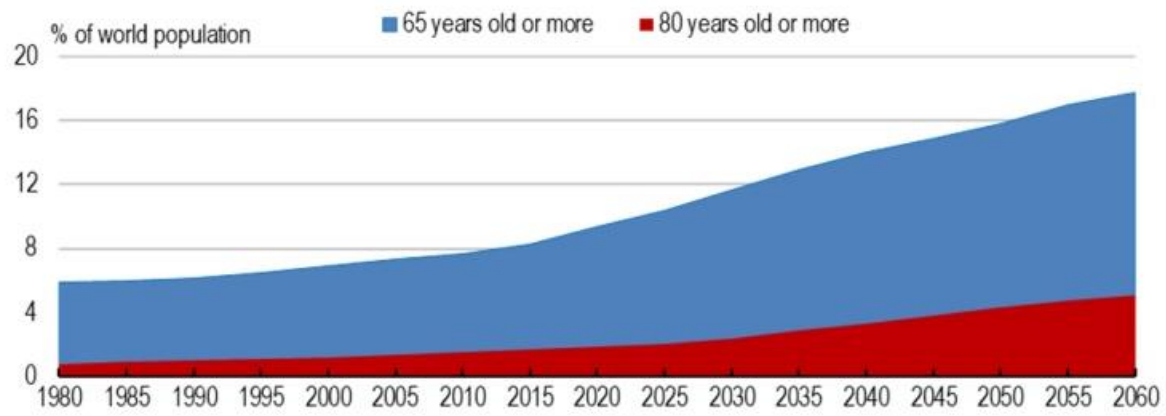

Figure 1.2: Increasing world's elderly population (United Nations, 2017)

1.0.1 Older Adults and Health

While aging is universal it is not identical, and the health status of older adults is impacted by several factors including psychological, socioeconomic, cultural beliefs and physiological changes (Hernández-Galiot & Goñi, 2017; De Morais et al., 2013). Living longer can present challenges related to health (Bloom et al., 2016b) and ageing is a principal risk factor for non-communicable chronic diseases such as diabetes, obesity, cardiovascular disease, Parkinson's disease, arthritis, anemia, urinary problems and cancer (Jura & Kozak, 2016; Thakur et al., 2013). As the ageing population increases so too is the prevalence of these diseases, with 62% of Americans aged 65+ diagnosed with more than one chronic condition (Jaul & Barron, 2017).

The Irish longitudinal Study on Ageing (TILDA) highlighted that chronic disease prevalence in Irish older adults dramatically transformed from 2009/2010 to 2016, with increased frequency of osteoporosis, cardiovascular disease, hypertension, lung disease, diabetes, heart attacks and arthritis (McNicholas & Laird, 2018). The increased frequency of these conditions translates to increased demands on our healthcare systems (Wren et al., 2017) thus it is of benefit for all societies to promote healthy ageing.

1.0.2 Diet and Healthy Ageing

Healthy ageing can be defined as prolonging the number of healthy active years which in turn extends life expectancy (Shlisky et al., 2017). The health of older adults can be heterogeneous in nature (Food Safety Authority of Ireland, 2000), however diet and nutrition have a major impact on healthy ageing (Jong et al., 2014; Mak & Caldeira, 2014) and identifying the nutritional needs of older adults is essential to maintain their quality of life, functional independence and promote healthy ageing (Leslie & Hankey, 2015).

Dietary needs change with age for several reasons. In general people become less active, metabolism slows down, and energy requirements decrease (Institute of Medicine (IOM), 2010). In 1999 Russell et al. published a food pyramid with the recommended dietary allowance (RDA) of different food groups for older adults (Figure 1.3) which was further modified in 2008 (Figure 1.4) by Lichtenstein et al. While there are some obvious differences between what is recommended for younger adults and older adults, there are some similarities, such as general daily requirements of vegetables, fruit, dairy and meat/protein quantities. Bauer et al. (2013) recommends that older adults consume 1.0-1.2g of protein per kilogram of body weight each day in order to remain healthy. Increased protein intake maintains functionality and nitrogen balance, enhances muscle strength, amplifies lean body mass and elevates recovery from illness (Van der Zanden et al., 2014; Bauer et al., 2013). Sugar/salt dense foods should be limited while fat intake should be regulated, decreasing saturated fats while increasing monounsaturated and polyunsaturated fats (Irish Nutrition & Dietetic Institute, 2019; Food Safety Authority of Ireland, 2000). Older adults should only consume dietary supplements, fortified foods and enriched foods following professional advice and depending on health status and individual needs (Food Safety Authority of Ireland, 2000). Older adults should also ensure to consume sufficient quantities of vitamins

(vitamin B12, vitamin D) and minerals (iron, calcium) (Irish Nutrition & Dietetic Institute, 2019; Food Safety Authority of Ireland, 2000).

Several factors can influence the probability of older adults consuming the RDA of essential food groups but the general tendency is consumption of insufficient quantities of whole grains, fruit, meat, poultry, vegetables and fish, while consuming excessive amounts of fried products, processed and sugary foods and refined grain products (Shlisky et al., 2017; Walton, 2011). Diet quality in older adult's decreases for a number of reasons, such as changes to appetite and decreased olfactory senses, oral health decline, poor mobility and sometimes decreased income can make it difficult for older adults to be able to access high quality foods (IOM, 2010). The demand for convenient, affordable food and healthy food and beverage options to support the energy requirements and health needs of older adult's is currently a key priority (Kramer et al., 2019; Giacalone et al., 2016; Schroll et al., 1996).

Food products targeted at older adults signifies an interesting segment of the global food industry and consumer market (Lutz et al., 2019). High quality and nutrient rich food products with attractive organoleptic properties are in high demand (Lutz et al., 2019).

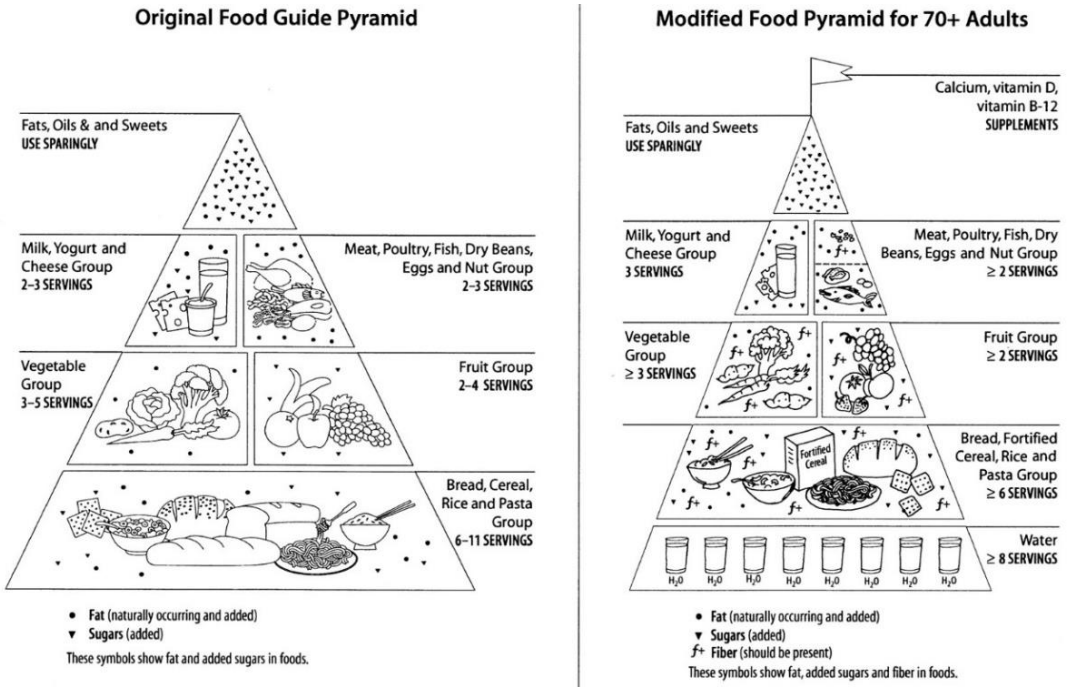

Figure 1.3: Modified Food Pyramid for 70+ Older Adults (Russell et al., 1999)

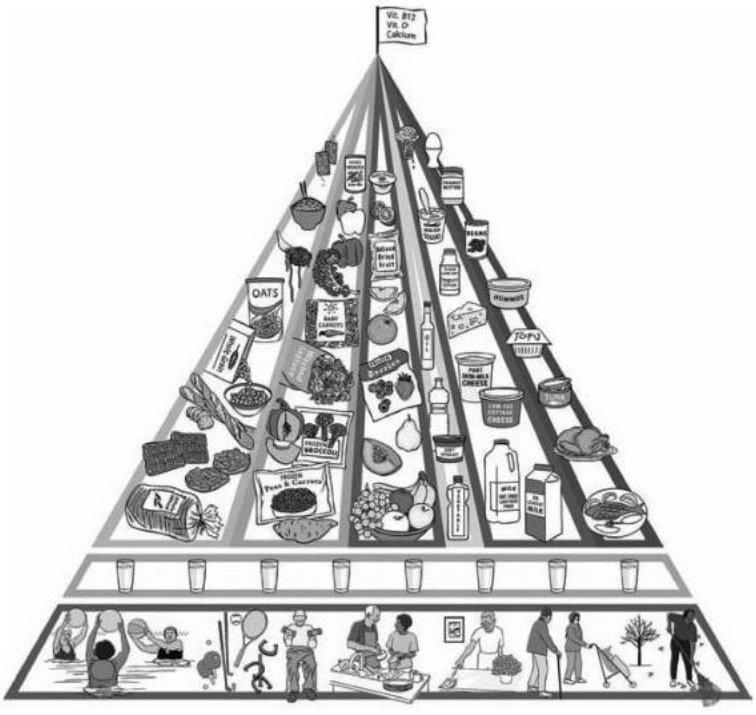

Figure 1.4: Modified Food Pyramid for Older Adults (Lichtenstein et al., 2008)

1.0.3 Potential Role of Functional Foods

Functional foods are food products that have enhanced natural health benefits in addition to their basic nutritional composition (Varzakas et al., 2018; Donato-Capel et al., 2014; Hasler, 2002). The concept of "functional foods" first came to surface in 1984 in Japan (Martirosyan & Singh, 2015) and within a short few years 1700 products were generated between 1988 and 1998 (Siró et al., 2008). Functional foods were originally developed to address nutritional deficiencies and deliver secondary metabolites that had known physiological and biological benefits, that could help defend against chronic diseases and infections (Motohashi et al., 2017; Chang, 2013). Functional food products can positively impact the immune system, reduce cardiovascular disease risk factors, improve intestinal function and delay cellular ageing by combating free radicals (Chiara et al., 2019). However, advise from qualified physicians and registered nutritionists is essential before any diet amendments are made, as mixing certain medication and functional foods might have detrimental effects (Motohashi et al., 2017).

Understanding the eating behaviours of target consumers as well as the factors that influence acceptance of novel food products is important in the design of functional foods (Van den Heuvel et al., 2019). The most common functional food products currently available on the market include cholesterol-lowering and vitamin D-enriched products, foods that incorporate probiotics and prebiotics and those that are omega-3-enriched and calcium-enriched (Taylor, 2011). Consumer acceptance is a vital element to functional food success and several factors can influence preferred choices including taste, functional food matrix, risk of adverse/negative effects, price, availability and bioactive ingredient perception (Vella et al., 2013; Urala & Lähteenmäki, 2004; Urala & Lähteenmäki, 2003). Health is recognised as a key motivator for functional food consumption and older adults recognise the relationship

between diet and health (Bhaskaran & Hardley, 2002). Enhancement in physical and mental performance, cosmetic and improvement of well-being have also been major influences (Vella et al., 2013). Studies suggest that older adults are interested in consuming functional foods that potentially address eye health, improve immunity and heart health, impact constipation/bowel health and address issues with diabetes, osteoporosis/bone health and cancer (Vella et al., 2013). Shatenstein et al. (2003) investigated the relationship between ageing and intake of functional foods and reported that women consumed a greater quantity of functional foods in comparison to men. Other studies have also reported that among older adults, females are more likely to consume functional foods (Herath et al., 2008; Verbeke, 2005; Frewer et al., 2003). This may be due to their attentiveness in relation to dietary habits, preparation and purchasing activities, as well as their food knowledge (Shatenstein et al., 2003).

Functional foods are widely consumed by the elderly (Chang, 2013) however food product structure, form and texture should be carefully considered in the design of functional foods for older adults (IOM, 2010). If the carrier product of the functional food is perceived as healthy, such as yoghurt, it is more likely to be accepted by consumers (Ares & Gámbaro, 2007).

In 2014, the fastest growing segment within the food industry were food products targeted at the elderly population (Goldman et al., 2014), with countries like the Netherlands, Germany, United Kingdom and France being top players within the functional food market (Siró et al., 2008). This sector of the food industry is exponentially growing (Phillips & Rimmer, 2013) and functional foods are now considered to have potential as treatment/promising solutions for chronic diseases and are an important factor in healthy ageing (Vella et al., 2013),

however proof of functionality through scientific research is essential for functional food development and marketing (Williams et al., 2006).

1.0.4 Health Benefits of Whole Grains

Barley, wheat, rye, oats and rice are common whole grains (Papageorgiou & Skendi, 2018; McRae, 2017; Arvanitoyannis & Tserkezou, 2008). Whole grains have many health benefits and are associated with a reduced risk of cancer, diabetes, obesity, inflammation and heart disease (Zhu, 2018; McRae, 2017; Shatenstein et al., 2003). Zong et al. (2016) reported that adding 16g more of whole grain to your daily diet lowered the risk of cancer, cardiovascular disease and improved mortality rates. Whole grains can compress factors like body weight, inflammation and blood pressure, as well as exert positive effects on blood lipid levels and hormonal and metabolic effects (O'Donovan et al., 2018).

Whole-grain-based products deliver health benefits beyond simple nutrients and aid in the reduction of disease development by means of their bioactive constituents like inulin, resistant starch, phenolics, tocopherols, dietary fibre, beta-glucan, carotenoids and tocotrienols (Sofi et al., 2019; Gong et al., 2018; Gani et al., 2012; Borneo & León, 2012; Bartlomiej et al., 2012). The carbohydrate, mineral, fat, vitamin and protein content of whole grains are necessary for health maintenance and support (Borneo & León, 2012). Whole grain phenolic content is parallel to that of vegetables and fruit (Gong et al., 2019). Whole grain cereal intake reduces the risk of obesity, strokes, diabetes, metabolic syndrome, cardiovascular disease, cancer and hypertension (Thies, 2017; McRae, 2017; Bultosa, 2016; Borneo & León, 2012; Shatenstein et al., 2003).

Europe has no standardized definition for whole grains (European Commission, 2019). Consequently, Ireland currently has no national guidelines or recommendations regarding

whole grain consumption and therefore recommended intake patterns are based on the 48 g/day US guideline (O'Donovan et al., 2018). While >91% of Irish adults consume whole grains daily, the mean intake of whole grains in the Irish population is currently only 27.8g per day, with only 19% of Irish adults reaching the US RDA (O'Donovan et al., 2018). This suggests that there is a need to promote whole grain intake in the Irish population, possibly by designing new and innovative food products.

1.1 Brewer's Spent Grain

1.1.1 Source

Over 800 million litres of beer is generated in Ireland annually, adding €1.7 billion to the Irish economy (Drinks Ireland, 2019). Waste minimisation and by-product management are the two main environmental pressures faced by breweries across Ireland today with sustainability and efficiency being their key priorities (Kerby & Vriesekoop, 2017). Brewer's Spent Grain (BSG) is a natural moist residual solid that is filtered out after the soaking of malt grain during the brewing process, with roughly 3.4 million tons generated in the EU every year (Mullen et al., 2015; Fărcaş et al., 2014b; Fărcas et al., 2013). BSG is the most abundant by-product of barley grain beer production (Lynch et al., 2016). As can be seen in Figure 1.5 and Figure 1.6, it is primarily composed of the remaining barley grain coverings, specifically husk, pericarp, aleurone and seed coat of the original barley grain (Connolly et al., 2013; Robertson et al., 2010).

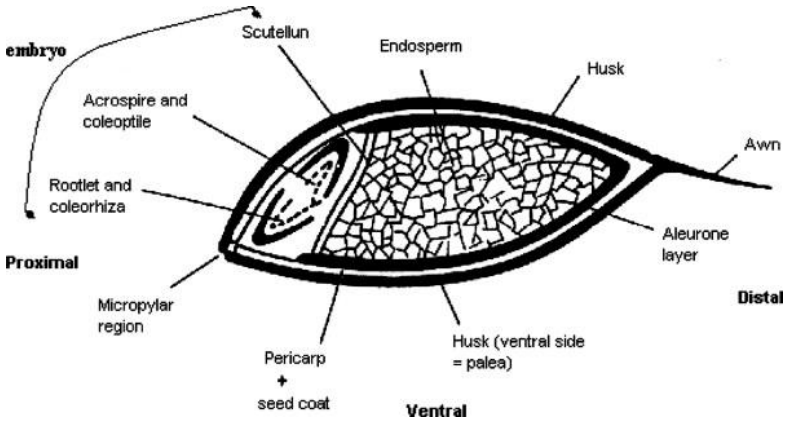

Figure 1.5: Barley kernel showing the grain coverings that constitute brewers' spent grains (Mussatto et al., 2006).

Figure 1.6: Image of dry BSG (Stojceska, 2011)

To obtain brewers spent grain, barley malt must be created first by passing the barley grain through five key steps (Figure 1.7), specifically cleaning/selection, steeping, germination, drying and storing (Xiros & Christakopoulos, 2012). The beer production process involves passing the barley malt through a range of stages including milling, mashing, lautering, boiling, wort separation, cooling, fermentation, maturation, pasteurisation, filtration, carbonation, cellaring and packaging (Lynch et al., 2016; Xiros & Christakopoulos, 2012). Enzymatic conversions take place during the mashing phase and during the lautering phase a sweet liquid known as wort is generated which consists of fermentable sugars and insoluble fractions (Xiros & Christakopoulos, 2012). These undegraded, solid fractions are the BSG

that settle at the bottom of the mash tun and the wort is allowed to filter through the BSG (Xiros & Christakopoulos, 2012).

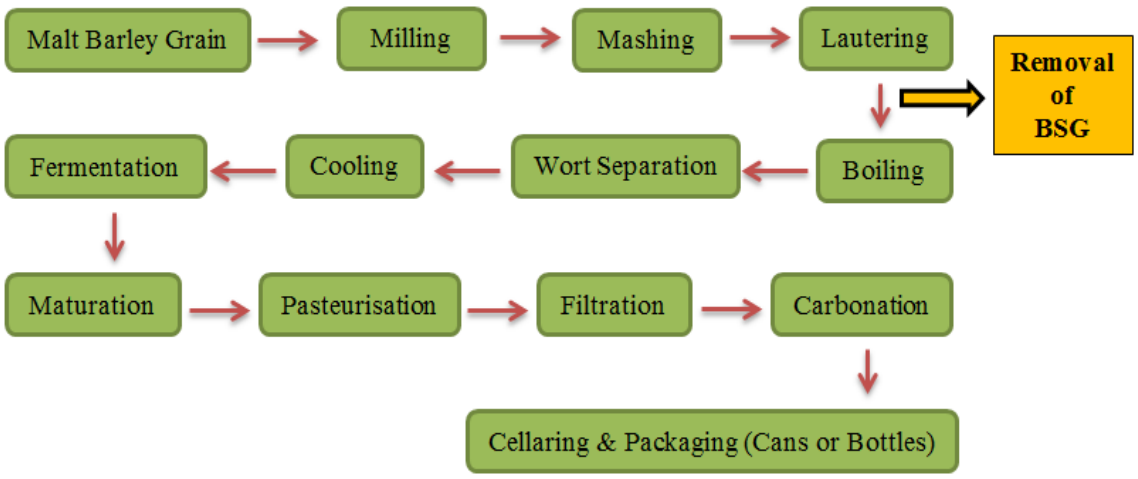

Figure 1.7: Principal Stages of Beer Production (adapted from: Lynch et al., 2016; Xiros & Christakopoulos, 2012)

BSG represents as much as 30% (w/w) of the initial malted grain (Fărcas et al., 2017). It has a high caloric value which enables it to be utilized as a substrate for biogas generation, biofuel combustion, production of construction bricks, growth medium for microorganisms and enzyme production, lactic acid production, metal adsorption and immobilization, hydroxycinnamic acids extraction, bioethanol production and xylitol and pullulan production (Flannery Nagel Environmental Ltd, 2016; Aliyu & Bala, 2011). BSG is primarily used in livestock feeding systems with roughly 93% incorporated into animal feed to provide the necessary protein for beef diets (Shen et al., 2019a; Jaiswal & Abu-Ghannam, 2014; Aliyu & Bala, 2011).

Of interest, BSG has a remarkable nutrition profile (Lynch et al., 2016) and several studies have investigated its health-promoting properties (Stojceska, 2019; Ikram et al., 2017; Steiner et al., 2015; Piggott & FitzGerald, 2014; Fărcaş et al., 2014b; Fărcaş et al., 2014a; McCarthy

et al., 2012; Salihu & Bala, 2011; Mussatto et al., 2006) and highlighted its potential as a functional food ingredient for human consumption (McCarthy et al., 2015; McCarthy et al., 2013d; McCarthy et al., 2013e; McCarthy et al., 2012). These studies suggest that this by-product may have as equal an economic importance as the main beer product (Buffington, 2014; Mussatto et al., 2006).

1.1.2 Composition

BSG is heterogeneous in nature, comprising the original barley grain seed coats, endosperm fragments, husk and pericarp (Steiner et al., 2015). BSG contains a wide range of phenolic compounds, protein, lipids and carbohydrates (Mathias et al., 2015) as well as polysaccharides and oligosaccharides (Mussatto et al., 2006). The barley grain husk segment of BSG is associated with a high phenolic yield (Fărcaş,et al., 2016).

BSG is a lignocellulosic material, consisting of protein (15-24%), cellulose (17-25%), lipids (10%), non-cellulosic carbohydrates (25-35%) lignin (8%-28%) and ash (5%). It also contains a range of minerals including sulphur, calcium, iron, sodium, cobolt, magnesium, selenium, copper, potassium, phosphorus and manganese, as well as B vitamins such as biotin, pyridoxine, folic acid, thiamine, niacin, choline, pantothenic acid and riboflavin (Mussatto et al., 2006).

1.2 Bioactive Components of BSG

Bioactive compounds are nonessential and essential food components found in food products in minor amounts that display positive therapeutic effects within cells, tissues or the entire body (Martín Ortega & Segura Campos, 2019; Hamzalıoğlu & Gökmen, 2016; Astley & Finglas, 2016). BSG is a valuable co-product with unexploited potential (Fărcas et al., 2013) and has been shown to contain highly desired bioactive compounds that include polyphenolic

compounds, bioactive peptides, polysaccharides and bioactive lipids (Lynch et al., 2016). In particular, BSG is known to be a rich source of antioxidant compounds (Verni et al., 2020; Cermeño et al., 2019; Bonifácio-Lopes et al., 2019; Stefanello et al., 2018; Parekh et al., 2017; Ikram et al., 2017; Fărcas et al., 2017; McCarthy et al., 2015; Reis and Abu-Ghannam, 2014a; Almeida et al., 2017; Fărcaş et al., 2015; McCarthy et al., 2013b; McCarthy et al., 2013d; McCarthy et al., 2013e).

1.2.1 Antioxidants

Antioxidants are represented by a distinctive group of enzymes and small organic molecules (Moussa et al., 2019; Romero et al., 2013) and are associated with decreasing oxidative stress-induced chronic diseases (Adefegha, 2018; Wen, 2020; Zhang et al., 2015b).

Oxidative stress is defined as an imbalance between levels of antioxidants and oxidants in the body, resulting in signalling disruptions that can translate to changes in physiological function (Sies, 2019; Tan et al., 2018; Pham-Huy et al., 2008) and modifications in the function and structure of nucleic acids, lipids and proteins, therefore leading to the development of diseases including diabetes, obesity, arthritis, cancer, vascular disease, osteoporosis, metabolic syndromes, Parkinson's, Alzheimer's, inflammatory bowel disease and atherosclerosis (Tan et al., 2018; He et al., 2017; de Araújo et al., 2016; Lobo et al., 2010; Pham-Huy et al., 2008). The body's oxido/redox balance is maintained by both antioxidant endogenous enzymatic mechanisms and exogenous antioxidant sources, including dietary antioxidants, which help to reduce damage to organs caused by radical nitrogen species (RNS) and radical oxygen species (ROS) by means of metal ion sequestration and affecting enzyme activities, gene expression regulation, hydrogen atom transfer, co-antioxidants and NO activity preservation (Hu et al., 2020; Santos-Sánchez, 2019; Moussa et al., 2019; Burton & Jauniaux, 2011; Lobo et al., 2010; Vaya & Aviram, 2001). Stability,

bioavailability, toxicity, pharmacokinetic character, potential synergistic behaviours, ROS nature and site of action are the key factors that impact antioxidant efficiency *in vivo* (Vaya & Aviram, 2001).

Antioxidants can be analysed based on oxidative initiation elimination or chain-breaking mechanisms through electron donation to electron acceptors or a particular ROS (Santos-Sánchez, 2019; Lobo et al., 2010; Vaya & Aviram, 2001).

From a food production point of view, antioxidant compounds are also known to preserve the nutritional quality of food products, delay toxic oxidation, boost product shelf life and decrease rancidity (Fukumoto & Mazza, 2000). Using natural antioxidants as food preservatives is an environmentally friendly, economical and sustainable approach to food production (Wen, 2020). Antioxidant peptides and phenolic compounds have been shown to reduce food oxidation (thus reducing quality losses such as flavour, colour, proteins and lipids) as well as promote health by reducing oxidative stress through a variety of promising mechanisms of action (Fukumoto & Mazza, 2000; Zhou & Yu, 2004; Wen, 2020; Miguel-Chávez, 2017).

1.2.2 Polyphenols

Polyphenols are secondary metabolites found in plant and grain food sources (Pandey & Rizvi, 2009; El Gharras, 2009). Polyphenols are natural and powerful antioxidants that have been shown to alleviate oxidative stress-induced damage and can play an essential role in promoting health and reducing disease risk (Salehi et al., 2020; Forni et al., 2019; Zhou et al., 2016; Li et al., 2016). Polyphenols include a variety of compounds specifically anthocyanins, stilbenes, flavonoids, tannins, coumarins, phenolic acids, omega-3 fatty acids, carotenoids, probiotics, phytoestrogens, limonoids, alkaloids, terpenoids, saponins and polyunsaturated acids (Adefegha, 2018; Zhou et al., 2016; Gupta et al., 2013). Flavonoids represent 60% of

all-natural polyphenols, while phenolic acids account for 30% (Zhou et al., 2016). A study conducted by Birsan et al. (2019) identified a total of fourteen different polyphenols in BSG. These compounds are associated with an array of biological functions that can support brain health, help maintain normal blood pressure and sugar levels, have neuroprotective features (against Parkinson's disease) and are anti-ageing, bacteriostatic, chemopreventive and anti-infective agents (Tian et al., 2019; Fraga et al., 2019; Adefegha, 2018; Gupta et al., 2013; Vauzour et al., 2010; Queen & Tollefsbol, 2010; Aquilano et al., 2008). Additional beneficial properties include cardio-protective, anti-inflammatory, anti-platelet, anti-diabetic, anticancer, anti-obesity and antioxidant traits (Cory et al., 2018; Pandey & Rizvi, 2009). Polyphenols have also been shown to promote lipolysis and decrease differentiation and adipocyte proliferation (Cory et al., 2018; Pandey & Rizvi, 2009). The metabolism of polyphenols can be influenced by other food matrix components for example polyphenol bioavailability can be altered when polyphenols interact with digestive enzymes and food proteins (El Gharras, 2009).

Currently, there are a variety of polyphenolic compounds that are known however, there is no recommended dietary intake (RDI) for these compounds. Subsequently there are no regulatory recommendations for polyphenol consumption in functional foods (Cory et al., 2018).

1.2.2.1 Hydroxycinnamic Acids

Phenolic acids are composed of at least one organic carboxylic acid joined to a phenol ring and can be classified as a hydroxycinnamic acid if it originates from cinnamic acid (Martini et al., 2019). Hydroxycinnamic acids within food influences more than one factor, for instance colour, odour, nutritional value, stability and flavour (Chen & Ho, 1997). Phenolic compounds and hydroxycinnamic acids have proven to act as potent antioxidants, possessing

significant beneficial and physiological functions in biological systems (Teixeira et al., 2013; Razzaghi-Asl et al., 2013). Sinapic, p-coumaric and ferulic acid are the three most abundant hydroxycinnamic acids in cereals and their health benefits rely on their bioavailability (El-Seedi et al., 2012).

Hydroxycinnamic acids are stored within the cell walls of the barley grain husk (Guido & Moreira, 2017; Fărcas et al., 2016) and 1g of BSG dry matter contains ~8mg of hydroxycinnamic acids (Lynch et al., 2016), with p-coumaric and ferulic acids being the most dominant (Dykes & Rooney, 2007; Bartolomé et al., 2002), followed by chlorogenic acid, caffeic acid, syringic acid and sinapic acid (McCarthy et al., 2012). As can be seen in Tables 1.1A–1.1E, the hydroxycinnamic acids present in BSG are associated with several health-promoting properties including anti-cancer, antimicrobial, anti-inflammatory, antioxidant and anti-atherogenic effects (Maróstica et al., 2010; Zhao et al., 2008).

Table 1.1A: Bioactive properties & health benefits linked to p-coumaric acid

Bioactive Properties	Health Benefits/ Functions	Reference
Antioxidant/ Free Radical Scavenging Power	**Maintenance of antioxidant enzymes** Glutathione (GSH), Superoxide Dismutase (SOD) & Catalase (CAT)	Ferreira et al., 2018 Boz, 2015b Abdel-Wahab et al., 2003
	Protection against oxidative damaged human lens epithelial cells by modulations in mitogen-activated protein kinases (MAPKs) signalling pathways	Peng et al., 2018
	Increase (↑) total antioxidant capacity & glutathione peroxidase activity	Shen et al., 2019b
Cardioprotective	Decrease **(↓)** low-density lipoprotein resistance, cholesterol oxidation & lipid peroxidation	Boz, 2015b
Anti-inflammatory	↓ Tumor necrosis factor alpha (TNF-α) expression *in vivo*	Pragasam et al., 2013
Relieve diabetic symptoms	Limits glucose metabolising enzymes ↑ Glucose transporter expression & beta-cell function	Pei et al, 2016 Amalan et al., 2016 Shairibha et al., 2014 Adisakwattana et al., 2004
Antimicrobial	Bind to the DNA of Shigella dysenteriae and trigger irreversible cell membrane permeability transformations	Lou et al.,2012
Renoprotective & Hepatoprotective	Protective effect against cisplatin (CIS)-induced oxidative damage in kidney and liver tissue *in vivo*	Ekinci Akdemir et al., 2017
Hypolipidemic	↓ Triglyceride levels, **l**ipid aggregation in hepatic tissue, steatosis of liver cells & upregulation of detoxifying enzymes	Shen et al., 2019b
Antimelanogenic	Aids in the prevention of cellular melanogenesis. Useful in the treatment of hyper-pigmentation	Seo et al., 2010 An et al., 2010
Antiplatelet qualities	Prevention of vascular and thrombotic diseases. Reduced adenosine diphosphate (ADP)-induced platelet aggregation	Luceri et al., 2007

Table 1.1B: Bioactive properties & health benefits linked to ferulic acid

Bioactive Properties	Health Benefits/ Functions	Reference
Antioxidant/ Free Radical Scavenging Power	Considered a super antioxidant: Easily absorbed, remains in the bloodstream for a long period of time & alleviates muscle fatigue by neutralising muscle tissue free radicals	Zduńska et al., 2018 Rukkumani et al., 2004
	Suppresses lipid peroxidation & binds to transition metals	
	Strengthens Superoxide Dismutase (SOD), Glutathione (GSH) and Catalase (CAT) activity	Kumar & Pruthi, 2014
Wound Healing	Improved wound contraction, period of epithelisation, as well as tensile strength *in vivo*	Dwivedi et al., 2015 Ghaisas et al., 2014
Relieve diabetic symptoms	Helps restore glucose homeostasis, suppresses gluconeogenesis, enhances hepatic glycogenesis and prevents insulin signalling negative regulators *in vivo*	Narasimhan et al., 2015
Antimicrobial	Inhibits the growth of *Pseudomonas aeruginosa, Shigella sonnei, Escherichia coli, Enterobacter aerogenes, Helicobacter pylori, Klebsiella pneumonia and Citrobacter koseri*	Boz, 2015a
	Assists in fungal conversion by functioning as a natural supply of vanillin, thus making it a desirable natural food preservative	Mandalari et al., 2005
Photoprotective agent	Skin protective: Shields elastin, fibroblasts, keratinocytes and collagen structures, advances wound healing, boosts angiogenesis and delays melanogenesis	Zduńska et al., 2018
Anti-wrinkle activity	Stimulated procollagen synthesis *in vitro*	Park et al., 2017
Hepatoprotective	Hinders the circulation of liver damaging markers *in vivo*	Rukkumani et al., 2004
	Diminishes oxidative stress *in vivo* by reducing serum, liver marker enzyme concentrations of γ-glutamyl transferase (GGT), alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP)	Shivashankara et al., 2015
Antiepileptogenic effect	Prevents seizure activity by enhancing gamma aminobutyric acid (GABA)ergic neurotransmission *in vivo*	Hassanzadeh et al., 2017

Table 1.1C: Bioactive properties & health benefits linked to sinapic acid

Bioactive Properties	Health Benefits/ Functions	Reference
Antioxidant/ Free Radical Scavenging Power	Metal-chelating properties demonstrated *in vivo*	Nićiforović & Abramovič, 2014 Pari et al., 2011
	Protection against lysosomal dysfunction and displays efficient free radical scavenging properties *in vivo*	Roy et al., 2012
	Supports the endogenous antioxidant defence system by replenishing catalase and other relevant proteins and reduces nitric oxide levels *in vivo*	Ansari et al., 2017
Anti-inflammatory	Reduces cytokine levels of interleukin 6 (IL-6), interleukin-1 β **(**IL-*1β)* & tumor necrosis factor alpha (TNF-α)	Ansari et al., 2017 Nićiforović & Abramovič, 2014 Cherng et al., 2013
Relieve diabetic symptoms	Improves hyperglycemia by boosting glucose utilization in diabetic rats through phospholipase C - protein kinase C (PLC-PKC) signals	Cherng et al., 2013
Cardioprotective	Prevents lipid peroxidation and demonstrates antioxidant behaviours in ex vivo rat heart preparations	Silambarasan et al., 2016 Silambarasan et al., 2015
	Shown to protect cardiac cell mitochondria by inhibiting deviations in membrane permeability	
	Played a role in arterial pressure reduction and kidney fibrosis inhibition	
Anti-cancer	Antiproliferative effects *in vitro*	Eroğlu et al., 2018 Kampa et al., 2004
Anti-thrombotic	Displays anti-coagulation and platelet aggregation inhibitory activities	Kim et al.,2016b
Protect the Brain	Neuroprotective properties against 6-hydroxydopamine (6-OHDA) neurotoxicity via reduction of nigral iron level and oxidative stress	Zare et al., 2014

Table 1.1D: Bioactive properties & health benefits linked to caffeic acid

Bioactive Properties	Health Benefits/ Functions	Reference
Antioxidant/ Free Radical Scavenging Power	Strong antioxidant characteristics at low concentrations, scavenges ROS species and impedes 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced lipid peroxidation	Maurya & Devasagayam, 2010
Cardioprotective agent	Constrained low-density lipoprotein oxidation	Olthof et al., 2001
	Inhibits hypertension-linked enzymes, such as angiotensin-converting enzyme (ACE), ectonucleoside triphosphate diphosphohydrolase (E-NTPDase), adenosine deaminase, (ADA) and (arginase)	Agunloye & Oboh, 2018
Anti-cancer	Protective role against oral, colon, liver, prostate and lung cancers	Tang et al. 2017 Magnani et al., 2014 Chiang et al., 2014 Lin et al., 2013
	Protective role against ultraviolet B (UVB)-induced skin damage, quenches cyclooxygenase-2 (COX-2) expression and the prostaglandin of E_2. More powerful anti-carcinogenic agent than chlorogenic acid.	Kang et al., 2009
	Shields phosphatidylcholine, a key lipid bilayer component of cell membranes from ultraviolet (UV) damage	Magnani et al., 2014
	Terminates tumor cells by mean of DNA oxidation, suppresses tumor growth by preventing expression of metalloproteases 2 and 9 and decreasing vascular endothelial growth factor induced vascularisation	Espíndola et al., 2019
Antimicrobial	Effective against *Staphylococcus aureus, Listeria monocytogenes, E.coli, Bacillus cereus* and some yeast species	Magnani et al., 2014
Protect the Brain	The blood-brain barrier is protected by both caffeic acid and caffeic acid phenethyl ester	Szwajgier et al., 2017 Zhao et al., 2012

Table 1.1E: Bioactive properties & health benefits linked to chlorogenic acid

Bioactive Properties	Health Benefits/ Functions	Reference
Antioxidant/ Free Radical Scavenging Power	Displays antioxidant activity against intestinal oxidative injury and ischemia–reperfusion injury in conjunction with shielding lipids, proteins and DNA from oxidative damage	Arauz et al., 2017
Relieve diabetic symptoms	Delays glucose release after a meal and expands glucose utilization in the liver	Garg, 2016 Meng et al., 2013
	Regulates type-2 diabetes by operating as an alpha-glucosidase inhibitor, decreases insulin spikes and is a glycaemic index lowering agent	
	Prevents glycogenolysis and gluconeogenesis by impeding the glucose-6-phosphatase enzyme; however, it can stimulate the 5' adenosine monophosphate-activated protein kinase (AMPK) enzyme which enables glucose utilization for muscle energy	Ong et al., 2012 Karthikesan et al., 2010
	Possesses antidiabetic qualities within streptozotocin-nicotinamide induced diabetic rats however a greater effect is accomplished when chlorogenic acid is in combination with tetrahydrocurcumin	
Anti-cancer	Suppresses cancer growth in the liver, large intestine and tongue	El-Seedi et al., 2012
	Protects DNA *in vitro* from impairment	Olthof et al., 2001
Protect the brain	Shown to improve the prefrontal brain function of elderly participants with subjective memory loss complaints. Has the ability to delay the onset of dementia	Kato et al., 2018
	Heightens memory and spatial learning	Cropley et al., 2011
	Encourages neuronal differentiation, delivers neuroprotective effects against oxidative stress and diminishes sleep latency. Enhances both attention control and motor speed.	Saitou et al., 2018

1.2.2.2 Lignans

Lignans are defined as fibre-associated, low molecular weight polyphenolic compounds and have been shown to be present in BSG (Yoder et al., 2015; Niemi et al., 2013a; Niemi et al., 2012b). Lignans have a phenylpropanoid core and classified as phytoestrogens (Simpson & Amos, 2017; Wcislo & Szarlej-Wcislo, 2014). Lignans are metabolised into mammalian lignans known as enterodiol and enterolactone by intestinal bacteria (Yoder et al., 2015) which participate in cytostatic activity against colon cancer (Wcislo & Szarlej-Wcislo, 2014). They possess antitumor, antihypertensive, anticancer, insecticidal, estrogenic, antiviral and antioxidant properties (Simpson & Amos, 2017). Secoisolariciresinol (SECO) and syringaresinol (SYR) are the most significant BSG lignans (Niemi et al., 2012b).

SECO exhibits a multitude of biological activities; however, a recent study conducted by Wang et al. (2013) demonstrated that SECO had effective antidepressant qualities whereby they can amplify brain monoamine levels (norepinephrine/noradrenaline and dopamine), highlighting SECO as a potential treatment option for menopausal-related depression. Tominaga et al. (2012) revealed that SECO administration compressed high-fat diet-induced obesity in C57BL/6 male mice by amplifying serum leptin production and weakening food intake, overall lowering weight gain. SECO has also been shown to decelerate the onset of type II diabetes and defend against lupus-induced renal function loss (Hosseinian et al., 2007).

Bajpai et al. (2018) established that SYR possessed anti-inflammatory properties, inhibiting lipopolysaccharide stimulated tumor necrosis factor-alpha, interleukin-6, prostaglandin E2, nitric oxide and interleukin-1beta in RAW 264.7 cells, making it a potential therapeutic treatment for inflammatory disorders. SYR has also proven to shield myocardial H9c2 cells against hypoxia/reoxygenation stimulated apoptosis, based on degradation of hypoxia-

inducible factor 1-alpha (HIF-1α) through forkhead box O3 (FOXO3) activation, thus making SYR an ideal therapeutic compound for ischemia-related diseases (Cho et al., 2015). Chung et al. (2012) reported that SYR encourages vasorelaxation by boosting endothelial cell nitric oxide (NO) generation via phosphatidylinositol 3-kinase/Akt- and calcium ions (Ca^{2+})/ phospholipase C (PLC)/ Ca(2+)/calmodulin-dependent protein kinase kinase-β (CaMKKβ)-dependent endothelial nitric oxide synthase (eNOS) phosphorylation and Ca^{2+}-dependent eNOS dimerization, highlighting SYR as a potential treatment for hypertension (Chung et al., 2012). A high-dose of SYR has also been shown to lower opportunistic pathogens like *Staphylococcaceae, Akkermansia* and *Bacteroidaceae* and strengthen *Bifidobacterium* and *Lactobacillus* populations (Cho et al., 2016).

1.2.3 Polysaccharides

BSG is abundant in non-cellulosic and cellulose polysaccharides, traces of starch, (1–3,1–4)-β-d-glucan and arabinoxylans, as well as monosaccharides, specially arabinose, glucose and xylose (Steiner et al., 2015).

1.2.3.1 β -glucan

β-glucan is a bioactive compound that originates in the cell walls of the barley endosperms (Miyamoto et al., 2018) and has been shown to be found in BSG (Lynch et al., 2016). β-glucan is reported to have a hypocholesterolemic effect by preventing cholesterol uptake within the intestine (Sofi et al., 2017; Drozdowski et al., 2010; Chen et al., 2010) and intensifying food bolus viscosity, which further impacts nutrient absorption, bile acid reabsorption, neutral sterols fecal excretion as well as bile acid production from cholesterol (Thandapilly et al., 2018; Chen et al., 2010). Its cholesterol lowering properties also reduce the risk of colorectal cancer, blood pressure, obesity and coronary heart disease (Bader Ul

Ain et al., 2018; Steiner et al., 2015; Stevenson & Inglett, 2009). Additional therapeutic benefits include, effective metabolism of sugars and fats (Sofi et al., 2017), promotion of wound healing, decreasing chronic fatigue syndrome, metabolically controlling diabetes through regulated glycemic responses, declining psychophysical stress and delaying cancer development (Sima et al., 2018; Bader Ul Ain et al., 2018). It also operates as an immune stimulant, affecting both adaptive and innate immunity, improving natural killer cell and macrophage function, amplifying immunoglobulin concentrations and boosting cellular and humoral immunological activity (Sima et al., 2018; Bader Ul Ain et al., 2018; Chan et al. 2009; Akramienė et al., 2007; Havrlentová & Kraic, 2006).

β-glucan also acts as a prebiotic within the gastrointestinal (GI) tract by means of gut microbial metabolite short-chain fatty acid generation in addition to its viscous nature, which not only results in advancements within postprandial glucose metabolism and insulin levels, but a suppressed appetite and reductions within serum cholesterol levels (Miyamoto et al., 2018; Mitmesser & Combs, 2017).

1.2.3.2 Arabinoxylans

The most abundant fibre rich, cereal grain cell wall polysaccharide are arabinoxylans (AX) with barley, rice, wheat, oats, sorghum and rye containing the highest quantity (Bastos et al., 2018; Cui et al., 2013). AX represents approximately 70% of the non-starch polysaccharides within cereal grain cell walls and alterations in structural features can greatly impact their physiochemical properties (Bader Ul Ain et al., 2018). BSG consists of ~22% of arabinoxylans (Coelho et al., 2016; Niemi et al., 2012b).

BSG contains this prebiotic performing, non-starch polysaccharide, which generates valuable bacterial metabolites, short-chain fatty acids (SCFA), including acetic, butyric and propionic

acid upon fermentation within the gut (Mendez-Encinas et al., 2019). Arabinoxylans promote gut health by encouraging the growth of favourable microflora (Sajib et al., 2018; Reis et al., 2014b; Mendis & Simsek, 2014), while amplifying microbial fermentation (Bader Ul Ain et al., 2018). Gómez et al. (2015) reported that arabinoxylooligosaccharides from Brewer's Spent Grain exhibited prebiotic activities, boosting lactobacilli and bifidobacteria populations and their functions, making it an applicable and beneficial prebiotic source in elderly nutrition and health. It can also reduce the accumulation of blood cholesterol by means of inhibiting the re-absorption of bile acids (Shelat et al., 2010). Arabinoxylans modify both the adaptive and innate immune system (Bader Ul Ain et al., 2018; Fadel et al., 2017; Zhang et al., 2015a).

Butyric acid energizes colonic epithelial cells (colonocytes) by functioning as a primary nutrient, with 90% being metabolized (Bedford & Gong, 2018). Butyric acid impacts a number of gut barrier functions including anti-inflammatory activity, gastrointestinal tract motility, immunoregulation, microflora composition and the life cycle of colonocytes (Załęski et al., 2013).

Propionic acid exhibits anti-bacterial and anti-fungal characteristics, overall impacting pathogenic bacteria colonisation, salmonella within the gastrointestinal tract (Al-Lahham et al., 2010). *In vivo* and *in vitro* human and animal studies have revealed that a range of immune cells and subsequent immune responses are influenced by cereal AX's (Bader Ul Ain et al., 2018).

The antioxidant activity of AX's is provided by the phenolic compounds within its structure, (Bader Ul Ain et al., 2018). The prebiotic nature of AX's brings about its cardioprotective properties (Bader Ul Ain et al., 2018).

1.2.4 Lipids

BSG lipids are dominated by triglycerides, subsequently free fatty acids (including linoleic VII oleic VI, palmitic), di-glycerides and monoglycerides (Del Río et al., 2013).

1.2.4.1 Oleic Acid

BSG contains oleic acid, the most abundant omega-9 monounsaturated fatty acid in nature, has shown to have a positive impact on cell membrane fluidity and is reported to play a significant role in cancer prevention, brain development, cardiovascular health, neuroregeneration, neuroprotection and decreased incidence of metabolic syndrome (Vázquez et al., 2019; Thavasiappan et al., 2016; Medina & Tabernero, 2010; Lopez-Huertas, 2010; Lopez et al., 2010; Bourre, 2009). Almeida et al. (2017) reported that BSG contained 103.71 ± 0.85 g kg-1 of oleic acid.

Oleic acid has the ability to reduce low-density lipoprotein (LDL) cholesterol and increase high-density lipoprotein (HDL) cholesterol, thus lowering blood pressure (Høstmark & Haug, 2013; Teres et al., 2008). Oleic acid also can diminish long-chain saturated fatty acid inflammatory properties in aortic endothelial cells (Carrillo et al., 2012) and appears to adjust $Ca2^{+}$ homeostasis within various immunocompetent cells, constrict Jurkat T cell proliferation and decline interferon gamma (IFN-γ) and interleukin-2 (IL-2) generation while weakening natural killer cell activity (Carrillo et al., 2012). Gonçalves-de-Albuquerque et al. (2016) established that oleic acid helps fight infections by lowering hepatic and kidney dysfunction markers. Palomer et al. (2018) reported that oleic acid possesses antidiabetic qualities, advances insulin sensitivity, develops β cell survival and in turn impedes endoplasmic reticulum (ER) stress. Oleic acid intake also raises the quantity of endogenous lipid mediator oleoylethanolamide which sequentially leads to reductions in body weight gain and food

intake as a result of peroxisome proliferator-activated receptor alpha (PPARα) activation (Palomer et al., 2018).

1.2.4.2 Stearic Acid

BSG also contains stearic acid (Niemi et al., 2012b); a nontoxic 18 carbon, plant and animal saturated fatty acid (Zhen et al., 2015). Stearic acid demonstrates a positive effect on LDL cholesterol, leading to improved heart function and decline in blood pressure and cancer probability (Senyilmaz-Tiebe et al., 2018). Stearic acid instigates mitochondrial fusion in human neutrophils and influences mitochondrial function (Senyilmaz-Tiebe et al., 2018). Almeida et al. (2017) reported that BSG contained 9.81 ± 0.15 g kg-1 of stearic acid.

Dietary stearic acid has proven to help regulate visceral adipose tissue, blood glucose and leptin concentration reduction, leading to a lower probability of diseases, such as metabolic syndrome, type-2 diabetes and cardiovascular disease (Shen et al., 2014). It can bring about cellular apoptosis; however, it is cell type, time and concentration dependent (Shen et al., 2014). Stearic acid had no negative effects on mature adipocytes or 3T3-L1 cell differentiation nonetheless amplified preadipocytes cytotoxicity and apoptosis (Shen et al., 2014). It has demonstrated beneficial inhibitory properties on metastasis, breast cancer growth, carcinogenesis and tumor growth (Shen et al., 2014) and has a positive effect on total body fat and abdominal fat levels (Shen et al., 2014).

1.2.5 Proteins and Peptides

BSG is a globulin, glutelin, albumin and hordein (>50% of total proteins) protein-rich source (Cermeño et al., 2019; Connolly et al., 2018; Vieira et al., 2017; Ikram et al., 2017) and has a higher protein concentration (26 - 30%) (Wen et al., 2019) in comparison to native barley as the starch segment is removed (Erasmus, 2009). BSG is composed of several essential amino

acids (~30%), specifically lysine, phenylalanine, threonine, methionine, leucine, isoleucine and tryptophan (Wen et al., 2019; Lynch et al., 2016; Essien & Udotong, 2008). Lysine is frequently scarce in cereal foods, thus highlighting the significance of BSG as a health-promoting ingredient (Lynch et al., 2016) and its protein hydrolysates as ideal protein source for the development of nutritional and functional food products (Wen et al., 2019).

BSG protein components are insoluble and must be enzymatically hydrolysed to improve its application within the food industry (Vieira et al., 2016; Niemi et al., 2013b; Celus et al., 2009). Cereals are protein rich sources and their peptides have been shown to have high *in vivo* and *in vitro* ACE inhibitory activity (Shamloo et al., 2015). Several studies have proven that BSG protein hydrolysates have a wide variety of associated bioactivities, such as angiotensin-I–converting enzyme inhibitory activity, anti-inflammatory and antioxidant properties (Wen et al., 2019; Connolly et al., 2016; Connolly et al., 2015; Connolly et al., 2014). The ACE I inhibitory activities of BSG protein hydrolysates enables BSG to be a safer preventive agent and more promising replacement to synthetic ACE drugs (Cian et al., 2018; Shamloo et al., 2015; Connolly et al., 2016; Iwaniak et al., 2014). Therefore, highlighting BSG as an ideal solution for elderly patients with hypertension as they exhibit vasodilatory and antihypertensive characteristics, providing good regulation of peripheral resistance as well as diastolic and systolic blood pressure (Ravid & Ravid, 1996).

The bioactive peptides of other cereals testify to possess anti-obesity, antiproliferative, anti-cancer, antithrombotic, immunomodulating, antimicrobial, pathogen infection protection and cholesterol-lowering properties (Malaguti et al., 2014; Cavazos & Gonzalez de Mejia, 2013; Mikušová et al., 2010), but this has not yet been explored within BSG.

1.3 BSG as a Functional Ingredient

Agricultural waste more than often consists of valuable by-products abundant in lipids, fibre and proteins, micronutrients and starch possessing desirable bioactivities, yet are more than often generally discarded (Faustino et al., 2019; Torres-León et al., 2018; Fărcaş et al., 2017). The potential application of agricultural by-products as functional ingredients for human nutrition is growing in attention. It would not only aid in the reduction and elimination of environment and economic concerns but would also contribute to the development of a diverse range of sustainable and innovative nutrient-rich food products (Torres-León et al., 2018; Steiner et al., 2015).

Alternative applications of BSG as a functional ingredient in value-added products is increasing and becoming more frequent (Chetrariu & Dabija, 2020; Steiner et al., 2015; Stojceska et al., 2008). BSG incorporation into food products is not only potentially beneficial for health but also practical and economical due to its' low cost and high levels of availability (Robertson et al., 2010). However, there are factors that must be considered with this strategy, such as quality, stability, sensory attributes, technological properties, functional characteristics, nutritional profile and active form of the individual active ingredient alone and within the desired food product (Day et al., 2009).

Several studies have attempted to incorporate BSG as a functional ingredient in food products, with varied success. BSG has been added to baked products, such as bread and breadsticks to enhance their nutritional profile. However, baking quality, rheological properties and texture can be impacted by its high fibre content (Nascimento et al., 2017; Ktenioudaki et al., 2013a; Ktenioudaki et al., 2013b; Ktenioudaki et al., 2012; Stojceska, 2011). Dietary fibre has the potential to increase up to fivefold when integrating up to 30% (w/w) of BSG during bread-making, but this amount of added fibre can negatively impact

dough development, shelf life, taste and water absorption in the product (Stojceska, 2011). Kissell & Prentice (1979) reported that an upper limit of 20% BSG incorporated into cookie products presented no concern in relation to flavour. However, Ajanaku et al. (2011) confirmed that 9-15% BSG supplemented cookie products received lower acceptance values for organoleptic properties in comparison to cookies encompassing 3-6% BSG. Iron and fat levels also increased with the addition of BSG in a food formulation, further enhancing the nutritional value (Ajanaku et al., 2011).

Further investigations carried out by Petrović et al. (2017) reported that cookie blends containing wheat flour and fresh, non-milled BSG at 15%, 25% and 50% provided high concentrations of protein, which altered colour development due to strong maillard reactions and had unpleasant texture. BSG's high fibre and protein content resulted in a great deal of water being absorbed which influenced additional product qualities including chewiness and hardness (Petrović et al., 2017). Samples encompassing 50% of BSG achieved the overall lowest acceptability while 25% samples attained the highest score for overall acceptability as well as taste and odour properties (Petrović et al., 2017), thus contrasting with the results obtained in the studies carried out by Kissell & Prentice (1979).

Cappa & Alamprese (2017) demonstrated that BSG can positively be utilized in fresh egg pasta products yet raw pasta quality is impacted, as lower dough elasticity, weaker gluten network formation and decreased firmness of fresh pasta was observed resulting in greater matter loss during cooking. However, Nocente et al., (2019) proved that BSG can enrich dry pasta, thus strengthening up to 135% fibre, 85% β-glucan and 57% resistant starch (20 g/100 g durum wheat semolina).

Most studies to date have concentrated on using whole/hydrolysed BSG, with a few studies using extracted BSG components. Spinelli et al. (2016) enriched fish products with

microencapsulated BSG polyphenols and flavonoids, and reported that this method was an effective way of disguising unpleasant flavours and aromas. The enriched fish products had 50% higher flavonoid and 30% greater phenolic content (Spinelli et al., 2016).

These studies have demonstrated the potential of BSG or its components to be successfully incorporated into food destined to for human consumption. BSG thus has potential as a functional food ingredient that can be added to a variety of food products to enhance the nutritional profile. However, the amount of BSG added must be carefully considered as high levels can negatively impact the organoleptic properties of the final product. Future research is necessary to generate BSG-based food products and confirm that the health properties associated with BSG-derived bioactive compounds are retained and effective *in vivo*.

1.3.1 Extraction Methods To Isolate BSG fractions

BSG is a complex matrix (Lynch et al., 2016). This research places a specific focus on the traditional and innovative extraction techniques applied for the recovery of novel and more potent BSG-derived fractions with improved bioactive potential.

Previous studies have employed a range of techniques to aid in component extraction and degradation of BSG. BSG polyphenolic and phenolic compounds have been specifically isolated and obtained via maceration, solid-phase extraction, ultrasound-assisted extraction, microwave-assisted extraction, soxhlet extraction, saponification, liquid–liquid extraction, supercritical carbon dioxide extraction, pressurized fluid extraction, supercritical fluid extraction, acid hydrolysis and solid–liquid extraction methods (Bonifácio-Lopes et al., 2019; Birsan et al., 2019; Stefanello et al., 2018; Carciochi et al., 2018; Guido & Moreira, 2017; McCarthy et al., 2012; Stalikas, 2007).

Microwave superheated water extraction, ultrasound assisted extraction and alkaline extraction method has been employed to obtain BSG arabinoxylo-oligosaccharides and arabinoxylans (Reis et al., 2015; Coelho et al., 2014). BSG protein concentrate is normally generated by subjecting BSG to chemical hydrolysis, alkaline extraction and acidification, followed by enzymatic hydrolysis of the BSG protein concentrate using enzymes like pepsin, flavourzyme and alcalase (Celus et al., 2007). Various pre-treatments such as hydrothermal, alkaline-acid sequential, one-step dilute acid, water-alkaline-acid sequential and alkaline pre-treatment involving ammonium carbonate have also been utilized to extract protein from BSG (Qin et al., 2018). Pre-treatments enhance the efficacy of the extraction method that follows, thus positively influencing the final product yield, reducing degradation compound development and overall decreasing operation cost and energy (Bonifácio-Lopes et al., 2019).

Overall, functional foods complement rather than substitute a healthy diet, yet their potential influence should not be overlooked as they present a range of bioactivities that support health and manage chronic diseases (Taylor, 2011).

1.3.2 In Vitro Digestion Models

In vitro digestion models are extensively used to investigate digestibility rates, structural changes that can occur to food components released from food matrices under gastrointestinal conditions (Hur et al., 2011). *In vitro* digestion models aim to mirror *in vivo* conditions, however the highly complex physiological and physiochemical events of *in vivo* systems makes it difficult to precisely replicate under *in vitro* conditions. *In vitro* models however, do pose less financial (less labour intensive and lower cost to run), ethical and technical (expensive and advanced instruments) challenges (Li et al., 2020; Brodkorb, et al., 2019; Minekus et al., 2014; Hur et al., 2011; Wickham et al., 2009). The *in vitro* model used may depend on the objectives of the study and models can differ in terms of mechanical stresses

(direction and magnitude), number of digestion steps included, buffer composition and incubation times. Models used can also reflect a specific target population of interest and therefore may consider characteristics such as health status, age and time of day (Santos et al., 2019; Hur et al., 2011).

The bioactivity, bioaccessibility and bioavailability of food-derived compounds will be influenced by the *in vitro* digestion models used (Gong et al., 2019; Alegría et al., 2015).

1.3.3 Bioactivity Investigations and In Vitro Cell-Based Studies

There is no accepted universal method by which antioxidant activity can be measured and there are a range of different chemical-based antioxidant assays that can be used. Assays which have previously been used to assess the antioxidant properties of BSG-derived fractions are based on different principles, and include the DPPH (2,2-diphenyl-1-picrylhydrazyl) Radical Scavenging, Ferric Reducing Antioxidant Power (FRAP) and Total Phenolic Content (TPC) assays (Ivanova et al., 2020; Andres et al., 2020; Birsan et al., 2019; Ivanova et al, 2017; Almeida et al., 2017; Reis & Abu-Ghannam, 2014a; Moreira et al., 2013; McCarthy et al., 2013d; McCarthy et al., 2012).

Total Phenolic Content is quantified spectrophotometrically using the Folin-Ciocalteu reagent (phosphomolybdic & phosphotungstic acid mixture), whereby in the presence of phenolic compounds under alkaline conditions changes colour from yellow to blue (tungsten-molybdenum) as a result of a oxidation/reduction reaction (Schendel, 2019; Lizcano et al., 2019; Hudz et al., 2019; Malta & Liu, 2014; Galili & Hovav, 2014; Sánchez-Rangel et al., 2013; Blainski et al., 2013; Huang et al., 2005).

The Ferric reducing antioxidant power of a sample is determined spectrophotometrically using the FRAP reagent, whereby in the presence of antioxidants under acidic conditions

(iron solubility preservation) reduces the colourless ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex to a blue ferrous tripyridyltriazine (Fe^{2+}-TPTZ) complex end product by means of electron donation (Bedlovičová et al., 2020; Ácsová et al., 2019; Rubio et al., 2016; Apak et al., 2016; Lim & Lim, 2013; Cerretani & Bendini, 2010; Huang et al., 2005).

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of a sample/extract is measured spectrophotometrically, whereby antioxidant hydrogen atom donation to DPPH radicals results in a reduced form (non-radical) with the loss of the deep violet colour of the DPPH regent to pale yellow, indicating the antioxidant concentration and ability to ability to quench the DPPH radical (Bedlovičová et al., 2020; Frezzini et al., 2019; de Torre et al., 2019; Ácsová et al., 2019; Apak et al., 2016; Gangwar et al., 2014; Kedare & Singh, 2011; Cerretani & Bendini, 2010).

It is recommended that multiple methods are used to assess the antioxidant properties of food-derived compounds and fractions (Prior et al., 2005). Other antioxidant assays include the Oxygen Radical Absorbance Capacity (ORAC) and ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) assay (Hu et al., 2020).

In vitro cell culture models are established from specific cell types, discrete organs or whole organisms and can be employed to analyse the effects of test compounds and fractions on cell differentiation and proliferation mechanisms, as well as apoptosis related events (Arango et al., 2013). Each cell culture model requires specific culture medium nutrients (e.g. fatty acids, inorganic salts, protein, vitamins, carbohydrates, amino acids, glucose) to survive and lower the risk of cell death and reduce slow cell growth, with O_2 and CO_2 levels being greatly considered (Verma et al., 2020; Arango et al., 2013; Selvakumaran & Jell, 2005). Reproducibility, consistency, suitability for high through-put testing and a reduction in the need for animal testing are the principal advantages and benefits of using cell culture models

(Aslantürk, 2018; Segeritz & Vallier, 2017; Arango et al., 2013). To ensure cell culture experiment conditions and reagents are precisely standardization it is necessary to accurately verify the number of viable cells first to highlight the quantity of healthy cells (Verma et al., 2020; Arango et al., 2013). Cell culture-based assays are classified based on end point measurements (luminescent, fluorescence, colour changes) and cell functions (enzyme activity, ATP production, nucleotide uptake activity, cell adherence, co-enzyme production, cell membrane permeability) (Aslantürk, 2018). In this study three cell culture models were selected to analyse the antioxidant, immunomodulatory and anti-obesogenic properties of BSG-derived fractions. The MTT assay is the most frequently employed *in vitro* cytotoxicity/cell viability assay and has been shown to have a high reproducibility, is both safe and straightforward and is more superior to dye exclusion assays (Aslantürk, 2018). The U937 cell line model is a human monocytic blood cell line that has been used in several studies to investigate the cellular antioxidant properties of food-derived bioactive fractions, including BSG-derived fractions (McCarthy et al., 2013b; McCarthy et al., 2013c; Cordier et al., 2013). The Jurkat T cell line is a human leukaemic T cell line often utilized for cytokine modulation investigations of bioactive compounds and has also been previously used to investigate the immunomodulatory properties of BSG-derived fractions (O'Sullivan et al., 2019; McCarthy et al., 2015; Gholijani et al., 2015; Crowley et al., 2015; McCarthy et al., 2013b; McCarthy et al., 2013c; Aherne & O'Brien, 2008). Finally, the murine embryo 3T3-L1 preadipocyte cell line is a model which has been previously used to analyse the potential of food-derived fractions to modulate adipocyte metabolism *in vitro* (Poulos et al., 2010).

Research Aims

What is evident from the literature is that BSG has a remarkable nutritional profile and is known to contain a variety of bioactive compounds. The antioxidant and anti-inflammatory properties of protein and phenolic BSG-derived fractions have been described previously.

The aim of this study is to generate novel BSG-derived fractions and, following *in vitro* digestion, investigate their potential antioxidant, immunomodulatory and anti-obesogenic properties using both cellular and non-cellular based assays. Fractions with greatest bioactivity will be identified for the purpose of being used as ingredients to develop novel functional foods for older adults and thus promote healthy ageing.

Chapter 2

In Vitro Digestion & Non Cellular Antioxidant Activity of BSG-Derived Fractions

Data from this chapter was presented at the 47th Annual Food Science and Technology Conference, University College Cork, December 2018

2.0 Abstract

The diet of older adults can play a significant role in promoting healthy ageing and, thus as the ageing population continues to increase, it is of interest to develop novel functional foods for this elderly cohort. Brewer's spent grain (BSG) is a by-product of the brewing industry and primarily used as animal feed. BSG is a protein and fibre-rich product and known to be a valuable source of bioactive compounds with diverse health promoting properties. Currently there is significant interest in identifying alternative applications for BSG and one strategy of interest is based on using BSG-derived ingredients to fortify human food products. This study focused on generating novel BSG-derived fractions and investigating their antioxidant properties following digestion using a simulated gastrointestinal *in vitro* digestion (SGID) model. The antioxidant potential of individual digestates and specific digestate combinations was examined by measuring total phenolic content (TPC), ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. In total 20 BSG-derived fractions were generated and 6 of these fractions were consistently associated with significantly higher ($p<0.05$) antioxidant activity compared to the intact BSG sample (CIT-1). Two of the novel protein-derived BSG fractions (CIT-4 and CIT-14) were associated with highest antioxidant properties. Combining digested protein and phenolic fractions was associated with predominantly antagonistic effects. BSG-derived fractions with improved antioxidant properties were identified. These fractions have a potential application as functional ingredients in healthy foods for older adults.

2.1 Introduction

Oxidative stress transpires when there is an imbalance between antioxidant defence and the accumulation of free radicals and oxidants, specifically reactive nitrogen species (RNS) or reactive oxygen species (ROS) (Pisoschi & Pop, 2015; López-Alarcóna & Denicola, 2013).

These compounds can negatively impact biological molecules by modifying protein and carbohydrate structures, causing amino acid oxidation and undesirable lipid peroxidation, they activate stress-induced transcription factors and transform nucleic acids and mitochondrial DNA structures (Birben et al., 2012). This cellular and tissue damage is linked to disease pathogenesis and elevates the ageing process (Fusco et al., 2007). In older adults oxidative stress is associated with the development of conditions such as type II diabetes, asthma, hypertension, osteoporosis, chronic obstructive pulmonary disease, atherosclerosis, Alzheimer's and cancer (Tan et al., 2018; Pisoschi & Pop, 2015; Birben et al., 2012).

While the effects of oxidative stress and free radical damage cannot be completely avoided, increasing levels of antioxidants can potentially minimise the effects (Lobo et al., 2010) and thus help to promote healthy ageing (Fusco et al., 2007). Antioxidants are substances that are found naturally in cells or can be sourced from the diet, and contribute to the complex signalling and metabolic mechanism that help to delay, constrict or eliminate oxidative damage (Wilson et al., 2017; López-Alarcón & Denicola, 2013; Fusco et al., 2007; Harman, 1995). Dietary sources of antioxidants include fruit, vegetables, nuts, dairy products, grains, legumes, meat, seafood, chocolate, tea and coffee (Carlsen et al., 2010). Several studies have reported on the potential role of dietary antioxidants in reducing the prevalence of age-related chronic conditions, including cardiovascular disease, hypertension, diabetes and cancer (Zhang et al., 2015b; Adefegha, 2018). A potentially effective strategy to promote healthy

ageing would be to increase dietary sources of antioxidants (Fusco et al., 2007) by designing functional foods for older adults that incorporate natural antioxidant ingredients (Jędrusek-Golińska et al., 2020). Functional foods are defined as processed or natural products that contain biologically active compounds that have additional health benefits beyond their basic nutritional composition (Wilson et al., 2017). Providing alternative healthy food choices for older adults that are rich in antioxidants would promote health in this increasing population (Wilson et al., 2017; Galanakis, 2017; Almeida et al., 2017; Bernstein & Munoz, 2012).

Brewers Spent Grain (BSG) is the most abundant by-product of barley grain beer production (Lynch et al., 2016), with almost 3.4 million tons produced annually in Europe by brewing industries (Ikram et al., 2017). BSG is a complex and unique residual solid fraction that has an interesting nutritional profile and is known to be an abundant source of bioactive compounds (Stefanello et al., 2018; Fărcas et al., 2017; Almeida et al., 2017; McCarthy et al., 2013a). BSG is a natural source of antioxidant-rich material and therefore, potentially an economical natural alternative to synthetic antioxidants (Fărcaş et al., 2015). The antioxidant properties of BSG have been extensively studied with a particular focus on its protein and phenolic components (Cermeño et al., 2019; McCarthy et al., 2013a; McCarthy et al., 2013e), however, lipids within the BSG structure are also associated with antioxidant properties (Parekh et al., 2017). The incorporation of whole BSG as an ingredient in formulated foods to increase levels of antioxidants has been reported (Reis and Abu-Ghannam, 2014a) and there is currently a lot of interest in improving extraction methods to isolate more potent bioactive BSG-derived fractions and further expand on this application (Ikram et al., 2017).

It is also worth noting that the potential health-promoting properties of food bioactivities are strongly related to their level of bioavailability (Azofeifa et al., 2018). The antioxidant properties of foods can change following digestion *in vivo*, which can influence the stability

of the structure and interactions between phytochemicals and other biomolecules in a food matrix and thus affect the bioaccessibility and bioavailability of bioactive compounds (Lucas-González et al., 2018). Several studies have used *in vitro* digestion models to assess the potential bioavailability of food-derived bioactives (Santos et al., 2019; Lucas-González et al., 2018). *In vitro* static digestion models are rapid and economical systems (Jones et al., 2019) that can differ in terms of enzymes used and digestion conditions (Alegría et al., 2015; Lucas-González et al., 2018) and serve as useful *in vitro* screening tools to investigate bioactivity.

Chemical-based antioxidant assays are cost effective screening tools (López-Alarcóna & Denicola, 2013), which can vary in experimental design and principle (Barros et al., 2017; Huang et al., 2005). Their application is useful, but it is recommended that more than one assay is applied to screen for antioxidant potential (Sunitha, 2016) as each biological antioxidant varies in terms of their structure, form and characteristics (Nagah & Seal, 2005). The majority of chemical-based antioxidant methods are built on the theory of radical species generation and the measurement of the radical inhibitory properties of the antioxidant, either been classified as indirect or direct assays (Nagah & Seal, 2005).

This study aimed to investigate the antioxidant properties of BSG and novel BSG-derived fractions, following *in vitro* simulated gastrointestinal digestion, using chemical-based antioxidant assays. Identification of potent antioxidant BSG-derived fractions could support the development of novel functional foods for older adults that would help promote healthy ageing.

2.2 Material and Methods

2.2.1 Materials

Reagents sourced from Sigma-Aldrich: 2,2-diphenyl-1-picrylhydrazyl (DPPH), Pepsin from Porcine Gastric Mucosa (Pepsin), (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), Bile extract porcine (Bile), Gallic Acid, Acetic Acid, Folin-Ciocalteau's Phenol Reagent, Iron (II) Sulfate Heptahydrate ACS reagent ≥ 99.0% (FeSO4.7H2O), 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ), Sodium Carbonate (Na_2CO_3), Sodium Chloride (Na_2Cl), Calcium Chloride ($CaCl_2$), Sodium Acetate (CH_3COONa), Magnesium Chloride ($MgCl_2$), Potassium Phosphate Monobasic (KH_2PO_4). **Reagents sourced from Fisher Scientific:** Sodium hydroxide (NaOH). **Reagents sourced from PanReac AppliChem (ITW Reagents):** Calcium Carbonate precipitated (USP, BP, Ph. Eur.) pure, pharma grade ($CaCO_3$). **Reagents sourced from Merck:** Sodium Hydrogen Phosphate (Na_2HPO_4), Magnesium Chloride Hexahydrate ($H_{12}Cl_2MgO_6$).

2.2.2 BSG Sample Preparation

In this study the potential bioactive properties of BSG (supplied by Diageo) were investigated by initially extracting and hydrolysing its varied components using a range of extraction procedures, digestive enzymes [carbohydrases, shearzyme, bioglucanase, proteases, biocellulase, alcalase (Alc) and flavourzyme (Fla)] and drying techniques, which are outlined in Figure 2.1. In total 20 BSG-derived fractions (CIT-2- CIT-21) were generated for analysis (conducted by University of Limerick project partners), and these together with the original intact BSG fraction (CIT-1) are listed in Table 2.1. Ultrafiltration membranes (3kDa, 10kDa and 50kDa) were used to obtain retentate and permeate BSG-derived fractions containing

different molecular weight compounds. Lab-scaled fractions were also performed at a semi-pilot scale. All lab-scaled and semi-pilot-scaled fractions were freeze-dried, with the exception of the semi-pilot scaled soluble, phenolic rich and hydrolysate samples which were spray-dried. Lab-scaled fractions were easier to freeze dry than spray dry mainly due to the small quantity of sample generated, as spray drying requires a large quantity of sample and is more cost effective for large scale operations. A downside to spray drying is that it only permits the use of soluble samples as otherwise the spray nozzle can be blocked.

Digestive enzymes were provided by Kerry Group and were also used during both lab and semi-pilot scaled methods in order to hydrolyse protein and carbohydrate components. Lab-scaled digestive enzymes included Carbohydrases (Shearzyme and Ultraflo). Semi-pilot scale digestive enzymes included three Carbohydrases (Bioglucanase FS2000, Bioglucanase HAB and Biocellulase) and two Proteinases (Alcalase and Flavourzyme). Each BSG-derived fraction was recovered twice by Dr. María Cermeño, University of Limerick using a diverse range of extraction methods.

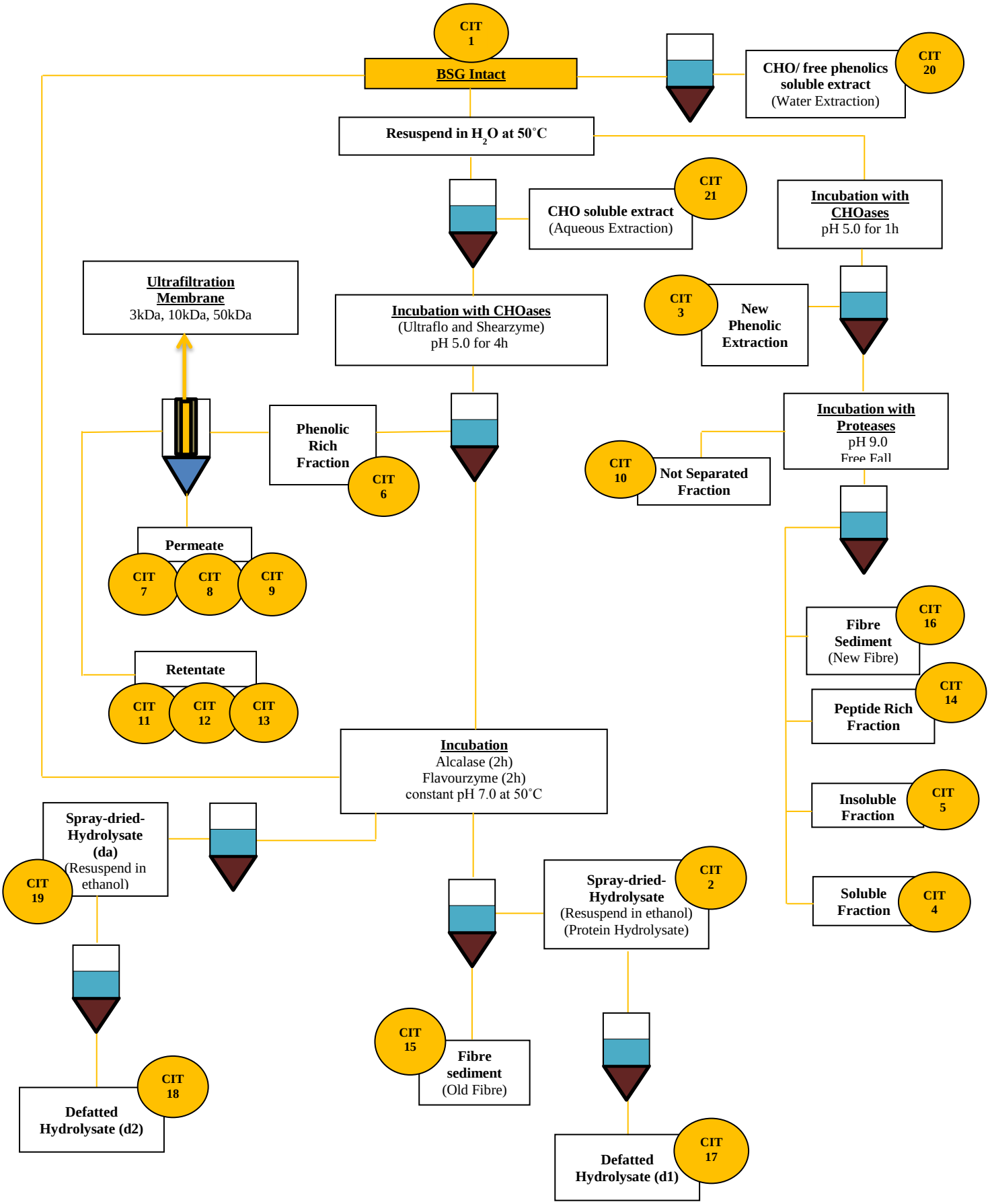

Figure 2.1: Recovery of BSG-derived fractions using a range of extraction methods involving different temperatures, enzymes and pH values (Procedures completed by Dr. María Cermeño, University of Limerick)

Table 2.1: BSG-derived fractions recovered following extraction procedures, assigned - Cork Institute of Technology (CIT) Code, quantities received and conditions of storage

BSG Fraction	CIT Code	Quantity Received	Storage
BSG Intact	CIT-1	3g	Room Temperature
Protein Hydrolysate	CIT-2	23g	Room Temperature
New Phenolic Extraction	CIT-3	-	Room Temperature
Soluble Fraction	CIT-4	2.96g	Room Temperature
Insoluble Fraction	CIT-5	2.98g	Room Temperature
Phenolic Rich Fraction	CIT-6	3.83g	(-20° C) Freezer
Permeate 3kDa	CIT-7	0.1716g	(-20° C) Freezer
Permeate 10kDa	CIT-8	0.1094g	(-20° C) Freezer
Permeate 50kDa	CIT-9	0.1304g	(-20° C) Freezer
Not Separated Fraction	CIT-10	3g	Room Temperature
Retentate 3kDa	CIT-11	0.591g	(-20° C) Freezer
Retentate 10kDa	CIT-12	0.2889g	(-20° C) Freezer
Retentate 50kDa	CIT-13	0.3143g	(-20° C) Freezer
BSG Peptide Rich Fraction	CIT-14	2.95g	Room Temperature
Old Fibre Rich Fraction	CIT-15	2.64g	Room Temperature
New Fibre Rich Fraction	CIT-16	3g	Room Temperature
Defatted Hydrolysate (d1)	CIT-17	-	Room Temperature
Defatted Hydrolysate (d2)	CIT-18	2.77g	Room Temperature
Hydrolysate (da)	CIT-19	6g	Room Temperature
Water Extraction	CIT-20	1g	Room Temperature
BSG Aqueous Extraction	CIT-21	3g	Room Temperature

2.2.3 In Vitro Digestion

All BSG-derived fractions were subject to an *in vitro* simulated gastrointestinal digestion (SGID) model that was representative of an adult digestive system and based on procedures described by Brodkorb et al. (2019) and Minekus et al. (2014) (Figure 2.2). Briefly, a 1% sample was prepared by mixing 0.1g of each BSG fraction in 10mls of sterile H_2O, sterile-filtered using a syringe filter (0.22μm) and stored in sterile eppendorf tubes at -20°C. For digestion, the gastric phase (Figure 2.2) was prepared by mixing 7.5mls Simulated Gastric Fluid (SGF) (Table 2.2), 2mls porcine pepsin enzyme (5mg/1ml SGF), 5μL of CaC_{l2} $(H_2O)_2$ (0.3M) and 0.295ml of H_2O. The pH of the mixture was adjusted to pH3 using HCL (6M) and incubated at 37°C for 2 hours in a shaking incubator. The pH of the gastric phase was monitored and maintained at pH3.

Table 2.2: Preparation of stock solutions of Stimulated Gastric Fluid (SGF) & Stimulated Intestinal Fluid (SIF)

Stock Solution	**Simulated Gastric Fluid SGF (ml)**	**Simulated Intestinal Fluid SIF (ml)**
KCl (0.5M)	**1.38**	**1.36**
KH_2PO_4 (0.5M)	**0.18**	**0.16**
$NaHCO_3$ (1M)	**2.5**	**8.1**
NaCl (2M)	**2.36**	**1.95**
$MgCl_2$ $(H_2O)_6$ (0.15M)	**0.08**	**0.22**
$(NH4)_2CO_3$ (0.5M)	**0.1**	**0**
H_2O	**73.4**	**68.21**
Total Volume (ml)	**80**	**80**

Following incubation, the sample was removed from the incubator and the intestinal phase conditions were initiated by adding 11ml of Simulated Intestinal Fluid (SIF), 5ml of pancreatin (1g/10ml SIF), 2.5ml of fresh bile (347.4mg/11ml SIF), 40μL of calcium chloride dihydrate (0.3M) and 1.31ml of H_2O. The pH of the mixture was adjusted to pH7 using

NaOH (1M) and incubated at 37°C for 2 hours in a shaking incubator. The pH of the intestinal phase mixture was monitored and if required, was readjusted to pH7 after the first hour of the incubation.

Following, digestion the samples were removed, centrifuged at 6,000g for 20mins using an ultracentrifuge (Beckman Coulter Ultracentrifuge Optima L-100XP). The supernatant was removed, sterile filtered (0.22µm) and aliquoted into sterile eppendorf tubes (1.5ml) and stored at -20°C until further analysed.

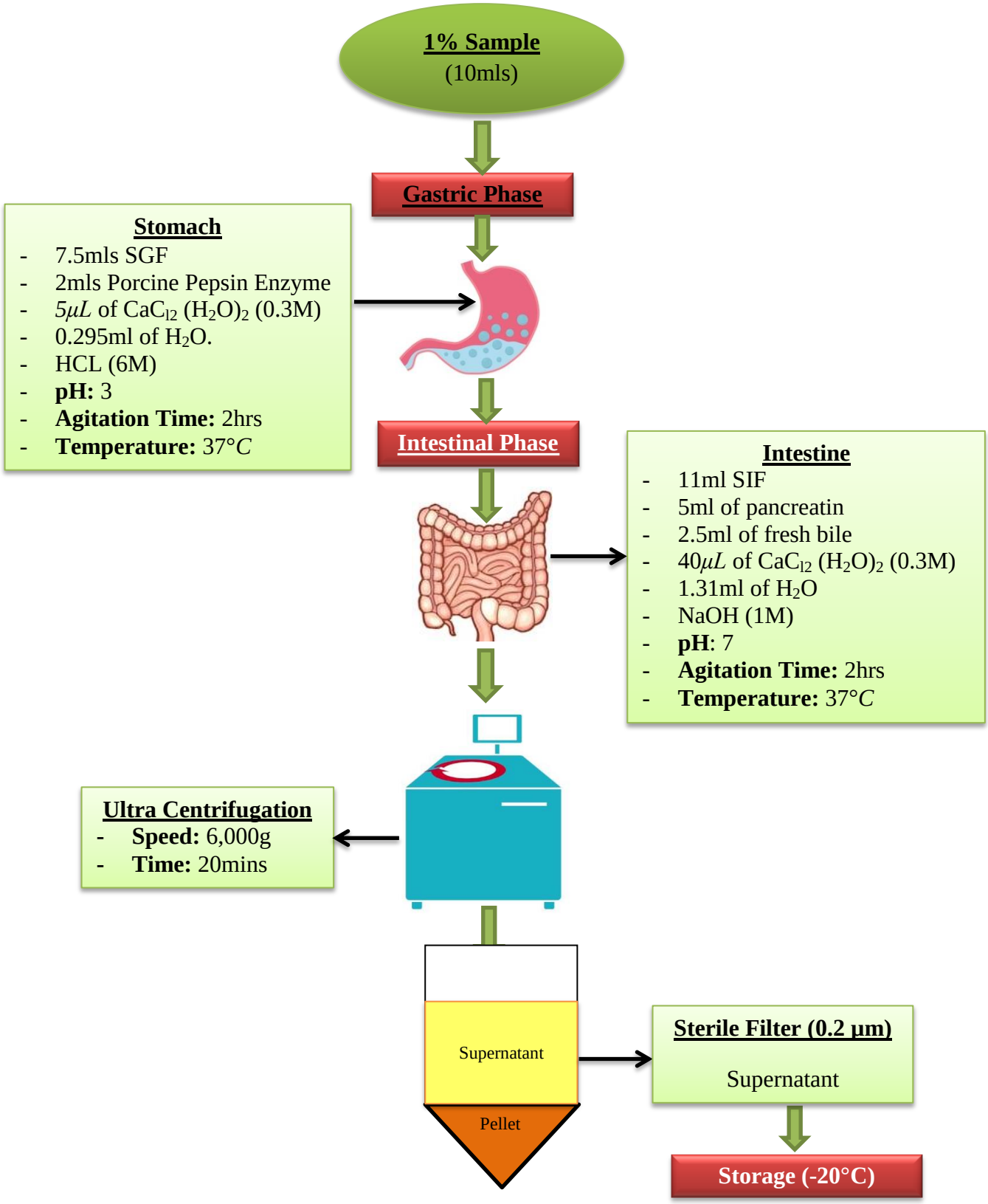

Figure 2.2: *In Vitro* Digestion Model representative of adult digestive system (adapted from Brodkorb, et al., 2019; Minekus et al., 2014)

2.2.4 BSG Blended/Combined Fractions

Digested BSG-derived protein and phenolic fractions (CIT-2 and CIT-6, respectively) were blended to examine if combinations of the digested fractions could promote a synergistic bioactive effect and increase the antioxidant potential of the BSG-derived fractions. The following protein: phenolic combinations were prepared based on the following ratios:

(i) **Combination 1 (C1)** - CIT-2 (10%) & CIT-6 (90%)
(ii) **Combination 2 (C2)** - CIT-2 (30%) & CIT-6 (70%)
(iii) **Combination 3 (C3)** - CIT-2 (50%) & CIT-6 (50%)
(iv) **Combination 4 (C4)** - CIT-2 (70%) & CIT-6 (30%)
(v) **Combination 5 (C5)** - CIT-2 (90%) & CIT-6 (10%)

Combinations were filter-sterilised using syringe filter (0.22μm) and stored in sterile eppendorf tubes at -20°C until further analysed (Figure 2.5 & Table 2.6).

2.2.5 Antioxidant Assays

In this study three antioxidant assays, Total Phenolic Content (TPC), Ferric Reducing Antioxidant Power (FRAP) and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, were employed to investigate the antioxidant potential of the individual BSG-derived fractions (pre- and post-digestion) and fraction combinations. Three independent experiments for each assay were set up and within each experiment each test sample was analysed in triplicate.

2.2.5.1 Total Phenolic Content (TPC) Assay

TPC was quantified according to the Folin-Ciocalteau's method (Abozed et al., 2014) with some modifications. This method involves the natural formation of a blue complex following the reduction of the Folin-Ciocalteau reagent by phenolic compounds, thus measuring the reducing capacity of a compound. In this study, 50µL of test sample was mixed with 250µL of Folin-Ciocalteu reagent before being vortexed for 3 secs. After 4 min incubation, the Folin-Ciocalteau reagent was neutralized with 500µL Na_2CO_3 and 4.2mls of deionised H_2O. The mixture was set aside in the dark for 2hrs at 25ºC. The absorbance of the reaction mixture was measured at 765nm against a blank (deionised H_2O) using a ultraviolet–visible (UV-Vis) Spectrophotometer (UV-1800, SHIMADZU EUROPA, Shimadzu Schweiz GmbH). The calibration curve of Gallic acid standards (0, 10, 20, 30, 40, 50mg/100mL) was plotted and the concentration of polyphenols in the BSG test samples was interpreted from the standard curve, with TPC expressed as milligrams of gallic acid equivalents per gram of dry weight sample (mg GAE g-1 ^{DW}). Precision was assessed by analysing each test sample in triplicate within each experiment and the experiment was repeated three times.

2.2.5.2 Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was completed based on the method originally described by Benzie & Strain (1999) and Benzie & Strain, (1996) with some modifications. This procedure is a reduction reaction in which a ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) is reduced to a blue coloured complex known as ferrous (Fe^{2+}-TPTZ), in the presence of antioxidants (Rajurkar & Hande, 2011). All solutions were prepared fresh on the day of the experiment. Briefly, to prepare the Standard Curve - FRAP reagent was formulated by mixing acetate buffer (300 mM, pH 3.6), 10mM TPTZ solution in 40 mM HCl and deionised water at 10:1:1 (*v*/*v*/*v*) respectively. For each Sample: FRAP reagent was formulated by mixing acetate buffer

(300 mM, pH 3.6),10 mM TPTZ solution in 40 mM HCl and 20 mM $FeCl_3$ at 10:1:1 (*v*/*v*/*v*) respectively. For analysis, 100 µL of each test sample was mixed with 900 µL distilled water and 2mL FRAP reagent. The reaction mixture was incubated in the dark at 25°C for 30 min prior to its absorbance detected spectrophotometrically at 593nm (UV-1800, SHIMADZU EUROPA, Shimadzu Schweiz GmbH). The standard curve was prepared using Trolox at different concentrations (0, 5, 10, 20, 40, 60, 80, 200 µmol) and the results were expressed as milligram of trolox equivalent (TE) per g test fraction. Analyses were performed in triplicate on each extract and the data presented represents the average of three experimental replicates.

2.2.5.3 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH free radical scavenging activity of all test fractions was assessed according to Szerlauth et al. (2019), Abozed et al. (2014), Ryan et al. (2011) and Brand-Williams et al. (1995) with some modifications. For this, 3.9mL DPPH/MetOH (0.06mM) was added to 100µL of the sample, mixed and the reaction solution was left to stand in the dark at 25°C for a 30min incubation period. A colour blank was used for all test fractions. Trolox was used as a standard (0.04, 0.08, 0.2 & 0.4µM). The absorbance was measured against a blank (methanol) at 517nm using a UV–Vis spectrophotometer (UV-1800, SHIMADZU EUROPA, Shimadzu Schweiz GmbH). A DPPH/methanol solution displays a strong purple colour due to delocalised electron of the DPPH radical, however when mixed with an antioxidant a yellow colour change appears, as a reduced form of DPPH transpires (DPPH-H) when the antioxidant hydrogen pairs up with the DPPH odd electron (Hangun-Balkir & McKenney, 2012). The inhibition concentration IC_{50} was determined by identifying the antioxidant concentration required to reduce the initial DPPH concentration by 50%, so therefore a high antioxidant activity would be represented by lower half maximal inhibitory concentration (IC_{50}) value (Pyrzynska & Pękal, 2013; Hangun-Balkir & McKenney, 2012).

Analyses were performed in triplicate on each extract and the data presented represents the average of three experimental replicates.

2.2.6 Statistical Analysis

Each BSG-derived fraction or combination (prior to and following *in vitro* digestion) was analysed in triplicate within each experiment and three independent experiments were conducted. Data presented represents the average ± standard deviation of all measurements. Statistical analysis was carried out using the IBM Statistical Package for the Social Sciences (SPSS v.26). A one-way analysis of variance (ANOVA) was used to compare differences in the bioactivity between all BSG-derived fractions following *in vitro* digestion. Controlling for multiple comparisons, the Dunnett's post-hoc test was used to evaluate mean changes between digestates and a control sample. A p-value <0.05 was considered statistically significant (See Appendices, Pages 208-211)

2.3 Results

2.3.1 Impact of digestion on antioxidant properties

TPC, FRAP and DPPH radical scavenging capacity of undigested and digested BSG-derived fractions CIT-2 and CIT-6, were compared to determine if digestion altered their antioxidant potential. Data indicated that the protein hydrolysate fraction (CIT-2) and the phenolic fraction (CIT-6) are antioxidant-rich fractions (Figure 2.3 and Table 2.3). *In vitro* digestion proved to negatively impact and significantly decrease ($p<0.05$) the antioxidant properties of CIT-2 across all three antioxidant assays (Figures 2.3A, 2.3B and 2.3C; Table 2.3). Similarly, for CIT-6, the digested fraction had significantly lower ($p<0.05$) FRAP and DPPH radical scavenging properties compared to the undigested fraction (Figures 2.3B & Figure 2.3C), but TPC was significantly higher ($p<0.05$) for the digested sample (Figures 2.3A; Table 2.3). The undigested protein fraction, CIT-2, exhibited greatest antioxidant activity across all three assays.

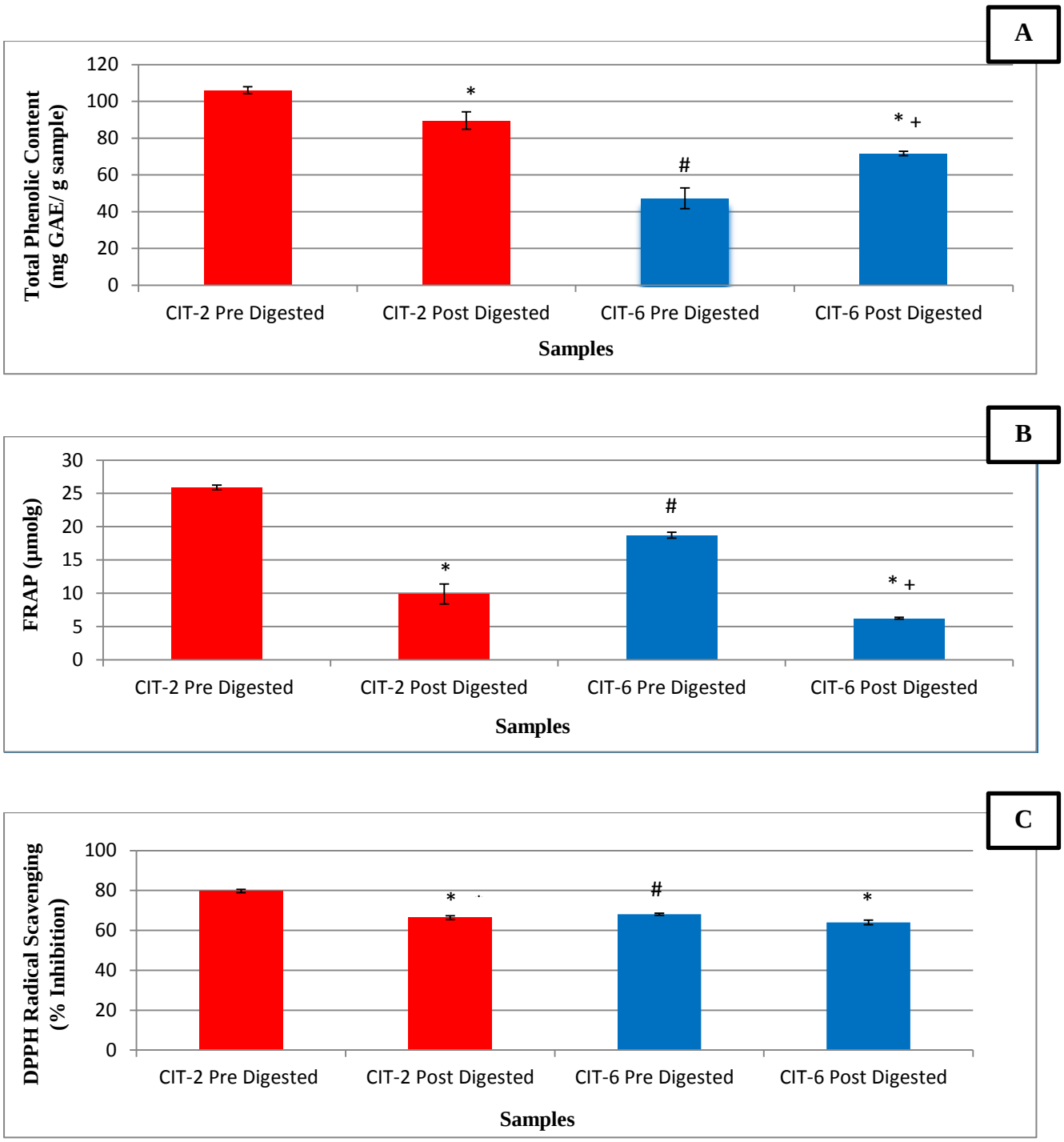

Figure 2.3: TPC/FCR Reducing Capacity (A); FRAP Activity (B); DPPH Scavenging Activity (C) of BSG CIT-2 & CIT-6 fractions prior to and following *in vitro* digestion. * denotes a statistically significant ($p<0.05$) difference between the pre and post digested values. # denotes a statistically significant ($p<0.05$) difference between the pre-digested phenolic and pre-digested protein values. + denotes a statistically significant ($p<0.05$) difference between the post-digested phenolic and post-digested protein values. Data represents mean ± standard deviation from three independent experimental replicates.

Table 2.3: Summary data of antioxidant potential, for CIT-2 & CIT-6 (pre- and post-digested). Data represents mean ± standard deviation (SD) values based on three independent experiments. ***** denotes a statistically significant ($p<0.05$) difference between the pre and post digested values. # denotes a statistically significant ($p<0.05$) difference between the pre-digested phenolic and pre-digested protein values. + denotes a statistically significant ($p<0.05$) difference between the post-digested phenolic and post-digested protein values.

Sample	TPC Assay Average (mg GAE/g sample ± SD)	FRAP Assay Average (µmol/g sample ± SD)	DPPH Assay Average (% Inhibition ± SD)
CIT-2 Pre Digested	106.104 ± 1.91	25.887 ± 0.35	79.724 ± 0.81
Post Digested	89.604 ± 4.72*	9.898 ± 1.50*	66.355 ± 0.95*
CIT-6 Pre Digested	47.302 ± 5.67#	18.719 ± 0.43#	68.143 ± 0.49#
Post Digested	71.660 ± 1.19*+	6.246 ± 0.13*+	63.993 ± 1.15*

2.3.2 Antioxidant properties of novel BSG-derived fractions

With an aim to isolate more potent BSG-derived fractions, novel extraction procedures were used to generate novel fractions. These fractions were digested and the antioxidant properties of the digestates were then compared to the BSG intact fraction (CIT-1), the protein hydrolysate fraction (CIT-2) and the main phenolic-rich fraction (CIT-6) to determine if the novel fractions had improved bioactivity.

TPC values of all BSG-derived fractions are presented in Figure 2.4A and Table 2.4 and as can be seen ranged from 53.315 ± 2.40 to 88.479± 8.04mg GAE/g sample. CIT-4, CIT-17 and CIT-14 had highest TPC values (88.479 ± 8.04mg, 88.121 ± 3.54mg, 87.143 ± 1.56mg GAE/g sample, respectively). CIT-14 and CIT-17 had significantly higher ($p<0.05$) TPC compared to CIT-1, CIT-2 and CIT 6, while CIT-4, CIT-18 and CIT-19 proved to have significantly higher ($p<0.05$) TPC compared to CIT-1 and CIT-6 but not CIT-2 (Figure

2.4A). Interestingly, CIT-15, CIT-20 and CIT-21 had significantly lower ($p<0.05$) TPC compared to CIT-1, CIT-2 and CIT 6 and were associated with lowest TPC (Figure 2.4A).

FRAP data for all BSG-derived fractions is presented in Figure 2.4B and Table 2.4 and as can be seen ranged from 4.283 ± 0.27 to 11.316 ± 0.60 µmol/g sample. CIT-2 had highest reducing potential (11.316 ± 0.60µmol/g), followed by CIT-17, CIT-12 and CIT-3 (10.761 ± 0.33µmol, 10.321 ± 0.45µmol, 10.159 ± 0.41µmol /g sample, respectively). The majority of the BSG-derived fractions (n=15 fractions) displayed significantly higher ($p<0.05$) FRAP compared to the intact BSG (CIT-1) (Table 2.5, Figure 2.4B). CIT-15 had a significantly lower ($p<0.05$) FRAP compared to fractions CIT-1, CIT-2 and CIT-6. Fraction CIT-17 exhibited a significantly higher ($p<0.05$) FRAP value than both CIT-1 and CIT-6 but had no significant difference to the antioxidant potential of CIT-2 (Figure 2.4B).

% DPPH inhibition data for all novel BSG-derived fractions is presented in Figure 2.4C and Table 2.4 and as can be seen ranged from -0.675 ± 1.48% to 11.814 ± 1.25 %. CIT-4, CIT-2 and CIT-14 displayed greatest DPPH radical scavenging activity (11.814 ± 1.25 %, 11.099 ± 0.80 %, 9.842 ± 0.96 % Inhibition, respectively). Twelve of the test fractions exhibited significantly higher ($p<0.05$) DPPH radical scavenging properties compared to the intact BSG fractions (Figure 2.4C). Fractions CIT-4 and CIT-14 presented significantly higher ($p<0.05$) DPPH radical scavenging activity compared to CIT-1 (intact BSG) and CIT-6 (phenolic-rich fraction) but had similar antioxidant potential to CIT-2 (protein hydrolysate). While fractions CIT-3 and CIT-18 had significantly higher ($p<0.05$) DPPH radical scavenging activity compared to CIT-1 and CIT-6, they had significantly lower ($p<0.05$) DPPH radical scavenging activity compared to CIT-2 (Figure 2.4C).

Overall, as can be seen in Table 2.4, six BSG-derived fractions (CIT-2, CIT-3, CIT-4, CIT-10, CIT-14 and CIT-18) were associated with significantly higher antioxidant activity compared to the BSG intact fraction (CIT-1) across all three assays, while eight fractions (CIT-6, CIT-7, CIT-8. CIT-9, CIT-11, CIT-13, CIT-17 and CIT-19) had significantly higher antioxidant activity across two of the antioxidant assays. Comparing the antioxidant activity of all BSG-derived fractions relative to each other (Table 2.5) confirmed that the extraction methods had major impact on the bioactivity of the fractions, with antioxidant properties significantly ($p<0.05$) altered.

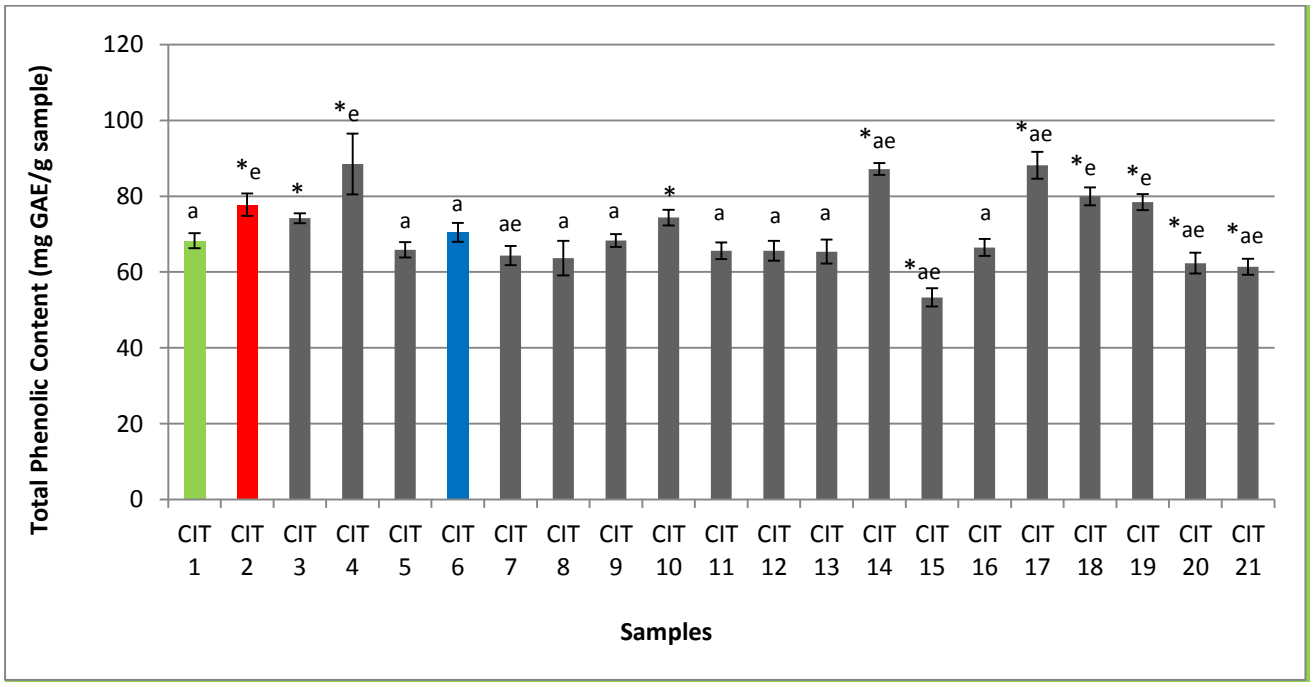

Figure 2.4A: TPC/Folin-Ciocalteu reagent (FCR) Reducing Capacity of BSG-derived fractions (CIT-1-CIT-21) following *in vitro* digestion. * denotes a statistically significant ($p<0.05$) difference between BSG intact fraction (CIT-1) and BSG-derived fractions. [a] denotes a statistically significant ($p<0.05$) difference between BSG protein fraction (CIT-2) and BSG-derived fractions. [e] denotes a statistically significant ($p<0.05$) difference between BSG phenolic fraction (CIT-6) and BSG-derived fractions. Data represents mean ± standard deviation from three independent experimental replicates.

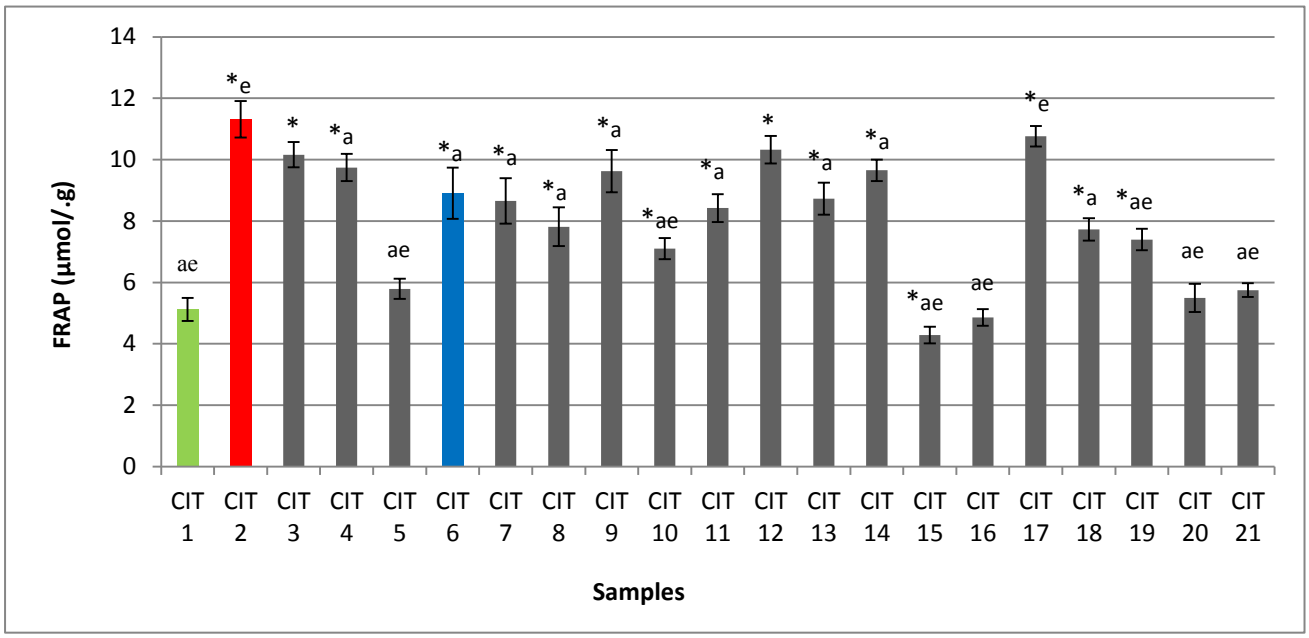

Figure 2.4B: FRAP Activity of BSG-derived fractions (CIT-1-CIT-21) following *in vitro* digestion. ***** denotes a statistically significant ($p<0.05$) difference between BSG intact fraction (CIT-1) and BSG digestate fractions. **[a]** denotes a statistically significant ($p<0.05$) difference between BSG protein fraction (CIT-2) and BSG-derived fractions. **[e]** denotes a statistically significant ($p<0.05$) difference between BSG phenolic fraction (CIT-6) and BSG-derived fractions. Data represents mean ± standard deviation from three independent experimental replicates.

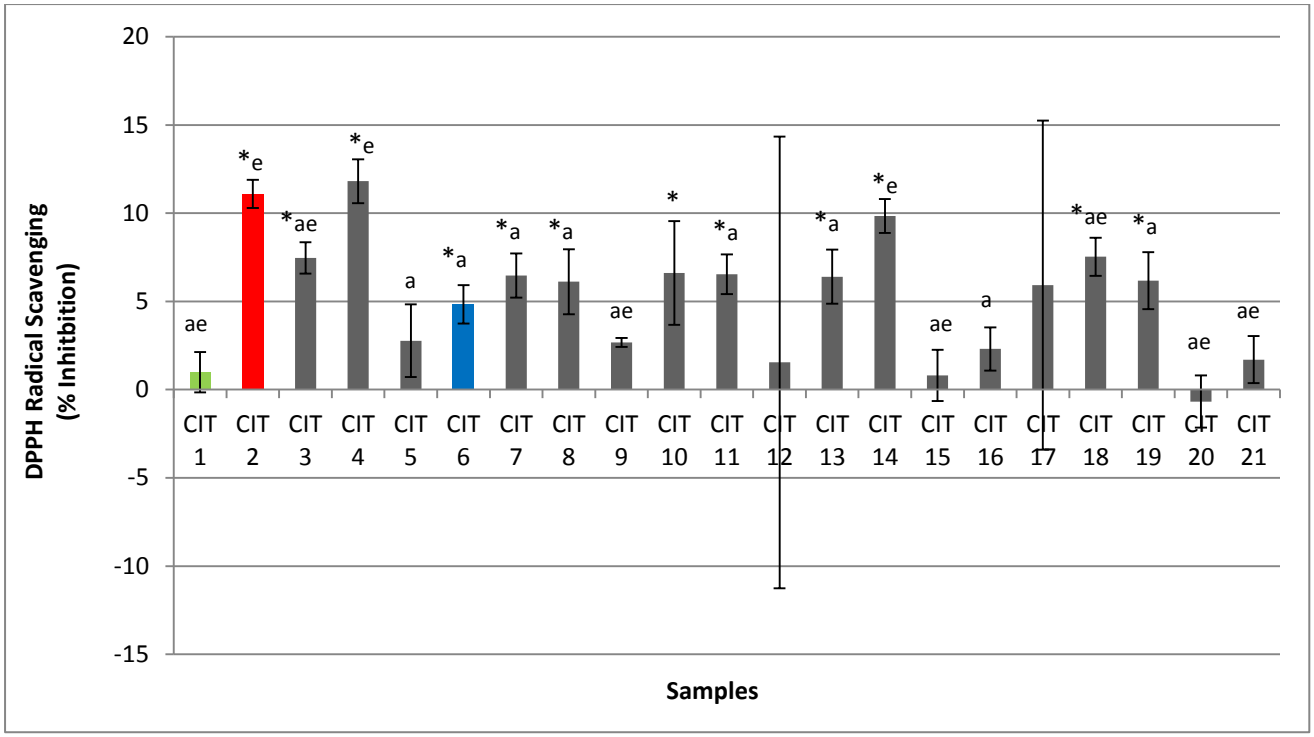

Figure 2.4C: DPPH Scavenging Activity of BSG-derived fractions (CIT-1-CIT-21) following *in vitro* digestion. ***** denotes a statistically significant ($p<0.05$) difference between BSG intact fraction (CIT-1) and BSG digestate fractions. **[a]** denotes a statistically significant ($p<0.05$) difference between BSG protein fraction (CIT-2) and BSG-derived fractions. **[e]** denotes a statistically significant ($p<0.05$) difference between BSG phenolic fraction (CIT-6) and BSG-derived fractions. Data represents mean ± standard deviation from three independent experimental replicates.

Table 2.4: Comparison of antioxidant properties for BSG-derived fractions (CIT-1-CIT-21) following *in vitro* digestion. Data represents mean ± standard deviation (SD) values based on three independent experiments. * Denotes a significant difference ($p<0.05$) in antioxidant activity, relative to BSG intact (CIT-1)/Sample Control. [a] denotes a statistically significant ($p<0.05$) difference between BSG protein fraction (CIT-2) and BSG-derived fractions. [e] denotes a statistically significant ($p<0.05$) difference between BSG phenolic fraction (CIT-6) and BSG-derived fractions.

CIT Code	TPC Assay Average (mg GAE/g sample ± SD)	FRAP Assay Average (µmol/g sample ± SD)	DPPH Assay Average (% Inhibition ± SD)
CIT-1	68.264 ± 1.95[a]	5.116 ± 0.38[ae]	0.981 ± 1.14[ae]
CIT-2	77.793 ± 2.95[*e]	11.316 ± 0.60[*e]	11.099 ± 0.80[*e]
CIT-3	74.190 ± 1.33[*]	10.159 ± 0.41[*]	7.465 ± 0.89[*ae]
CIT-4	88.479 ± 8.04[*e]	9.743 ± 0.45[*a]	11.814 ± 1.25[*e]
CIT-5	65.829 ± 2.02[a]	5.791 ± 0.33[ae]	2.773 ± 2.05[a]
CIT-6	70.483 ± 2.47[a]	8.905 ± 0.83[*a]	4.836 ± 1.10[*a]
CIT-7	64.325 ± 2.54[ae]	8.655 ± 0.74[*a]	6.461 ± 1.25[*a]
CIT-8	63.700 ± 4.56[a]	7.814 ± 0.63[*a]	6.113 ± 1.85[*a]
CIT-9	68.286 ± 1.68[a]	9.628 ± 0.69[*a]	2.675 ± 0.26[ae]
CIT-10	74.361 ± 2.06[*]	7.098 ± 0.35[*ae]	6.616 ± 2.94[*]
CIT-11	65.628 ± 2.21[a]	8.423 ± 0.45[*a]	6.538 ± 1.13[*a]
CIT-12	65.640 ± 2.61[a]	10.321 ± 0.45[*]	1.542 ± 12.80
CIT-13	65.381 ± 3.18[a]	8.731 ± 0.52[*a]	6.403 ± 1.54[*a]
CIT-14	87.143 ± 1.56[*ae]	9.655 ± 0.35[*]	9.842 ± 0.96[*e]
CIT-15	53.315 ± 2.40[*ae]	4.283 ± 0.27[*ae]	0.809 ± 1.46[ae]
CIT-16	66.482 ± 2.23[a]	4.859 ± 0.27[ae]	2.309 ± 1.23[a]
CIT-17	88.121 ± 3.54[*ae]	10.761 ± 0.33[*e]	5.925 ± 9.33
CIT-18	79.960 ± 2.34[*e]	7.724 ± 0.36[*a]	7.533 ± 1.08[*ae]
CIT-19	78.439 ± 2.14[*e]	7.398 ± 0.35[*ae]	6.180 ± 1.62[*a]
CIT-20	62.337 ± 2.74[*ae]	5.498 ± 0.46[ae]	-0.675 ± 1.48[ae]
CIT-21	61.359 ± 2.11[*ae]	5.749 ± 0.23[ae]	1.703± 1.34[ae]

Table 2.5: Comparison of antioxidant properties of all BSG-derived fractions relative to each other (CIT-2-CIT-21)/Multiple Comparisons following *in vitro* digestion. Data represents mean ± standard deviation (SD) values based on three independent experiments. Fractions with significantly ($p<0.05$) different antioxidant activity are differentiated by superscript letters (a-t), with the following associations: **CIT-2**=a; **CIT-3**=b; **CIT-4**=c; **CIT-5**=d; **CIT-6**=e; **CIT-7**=f; **CIT-8**=g; **CIT-9**=h; **CIT-10**=i; **CIT-11**=j; **CIT-12**=k; **CIT-13**=l; **CIT-14**=m; **CIT-15**=n; **CIT-16**=o; **CIT-17**=p; **CIT-18**=q; **CIT-19**=r; **CIT-20**=s; **CIT-21**=t

CIT Code	TPC Assay Average (mg GAE/g sample ± SD)	FRAP Assay Average (µmol/g sample ± SD)	DPPH Assay Average (% Inhibition ± SD)
CIT-2	77.793 ± 2.95^{defghjklmnopst}	11.316 ± 0.60^{cdefghijlmnoqrst}	11.099 ± 0.80^{bdefghjlnoqrst}
CIT-3	74.190 ± 1.33^{cdfghjklnopqrst}	10.159 ± 0.41^{dfgijlnoqrst}	7.465 ± 0.89^{acdehmnost}
CIT-4	88.479 ± 8.04^{abdefghijklmnost}	9.743 ± 0.45^{adgijnopqrst}	11.814 ± 1.25^{bdefghijlnoqrst}
CIT-5	65.829 ± 2.02^{abcimnpqr}	5.791 ± 0.33^{abcefghijklmnopqr}	2.773 ± 2.05^{abcjmq}
CIT-6	70.483 ± 2.47^{acmnpqrst}	8.905 ± 0.83^{adinoprst}	4.836 ± 1.10^{abchmnqst}
CIT-7	64.325 ± 2.54^{abceimnpqr}	8.655 ± 0.74^{abdiknopst}	6.461 ± 1.25^{achmnost}
CIT-8	63.700 ± 4.56^{abcimnpqr}	7.814 ± 0.63^{abcdhkmnopst}	6.113 ± 1.85^{achmnost}
CIT-9	68.286 ± 1.68^{abcimnpqrst}	9.628 ± 0.69^{adginoqrst}	2.675 ± 0.26^{abcefgjlmqrs}
CIT-10	74.361 ± 2.06^{cdfghjklmnopqst}	7.098 ± 0.35^{abcdefhjklmnopst}	6.616 ± 2.94^{cns}
CIT-11	65.628 ± 2.21^{abcimnpqr}	8.423 ± 0.45^{abcdikmnoprst}	6.538 ± 1.13^{acdhmnost}
CIT-12	65.640 ± 2.61^{abcimnpqr}	10.321 ± 0.45^{dfgijlnoqrst}	1.542 ± 12.80
CIT-13	65.381 ± 3.18^{abcimnpqr}	8.731 ± 0.52^{abdiknoprst}	6.403 ± 1.54^{achmnost}
CIT-14	87.143 ± 1.56^{abdefghijklnoqrst}	9.655 ± 0.35^{adgijnopqrst}	9.842 ± 0.96^{bdefghjlnorst}
CIT-15	53.315 ± 2.40^{abcdefghijklmnopqrst}	4.283 ± 0.27^{abcdefghijklmnpqrst}	0.809 ± 1.46^{abcefgijlmqr}
CIT-16	66.482 ± 2.23^{abcimnpqrt}	4.859 ± 0.27^{abcdefghijkmpqrt}	2.309 ± 1.23^{abcfgjlmqr}
CIT-17	88.121 ± 3.54^{abdefghijklnoqrst}	10.761 ± 0.33^{cdefgijlmnoqrst}	5.925 ± 9.33
CIT-18	79.960 ± 2.34^{bdefghijklmnopst}	7.724 ± 0.36^{abcdhkmnopst}	7.533 ± 1.08^{acdehnost}
CIT-19	78.439 ± 2.14^{bdefghjklmnopst}	7.398 ± 0.35^{abcdehjklmnopst}	6.180 ± 1.62^{achmnost}
CIT-20	62.337 ± 2.74^{abcehimnpqr}	5.498 ± 0.46^{abcefghijklmnpqr}	-0.675 ± 1.48^{abcefghijlmqr}
CIT-21	61.359 ± 2.11^{abcehimnopqr}	5.749 ± 0.23^{abcefghijklmnopqr}	1.704 ± 1.34^{abcefgjlmqr}

2.3.3 Effect of combining BSG fractions

To determine if combining BSG-derived fractions had a synergistic effect on antioxidant potential, various combinations of CIT-2 and CIT-6 were prepared and analysed. As can be seen in Figure 2.5A, none of the combinations (C1-C5) significantly increased TPC compared to the individual digested fractions. Indeed, TPC for combinations C1 and C2 was significantly lower ($p<0.05$) than both individual digested fractions. Data indicated that as the amount of protein in the combination increased so too did TPC. Similarly, DPPH data demonstrated that combining CIT-2 and CIT-6 (in any combination) did not have a synergistic effect on radical scavenging properties with all 5 combinations demonstrating significantly lower ($p<0.05$) activity. The individual CIT-2 and CIT-6 digestates demonstrated highest DPPH inhibition (66.355% and 63.993 %, respectively).

Contrary to TPC and DPPH, the reducing power of the combinations, as measured by FRAP, significantly increased ($p<0.05$) compared to the individual digestates, suggesting a synergistic effect (Figure 2.5B; Table 2.6). CIT-6 digestate demonstrated the weakest FRAP (6.246 ± 0.13 µmol/g sample), while combination C5 (90% CIT-2, 10% CIT-6) exhibited highest FRAP (25.614 ± 0.14 µmol/g sample). It is worth noting that the antioxidant potential of the combinations improved when the percentage of the protein hydrolysate fraction (CIT-2) was increased, with the most antioxidant combination based on a higher protein: phenolic ratio.

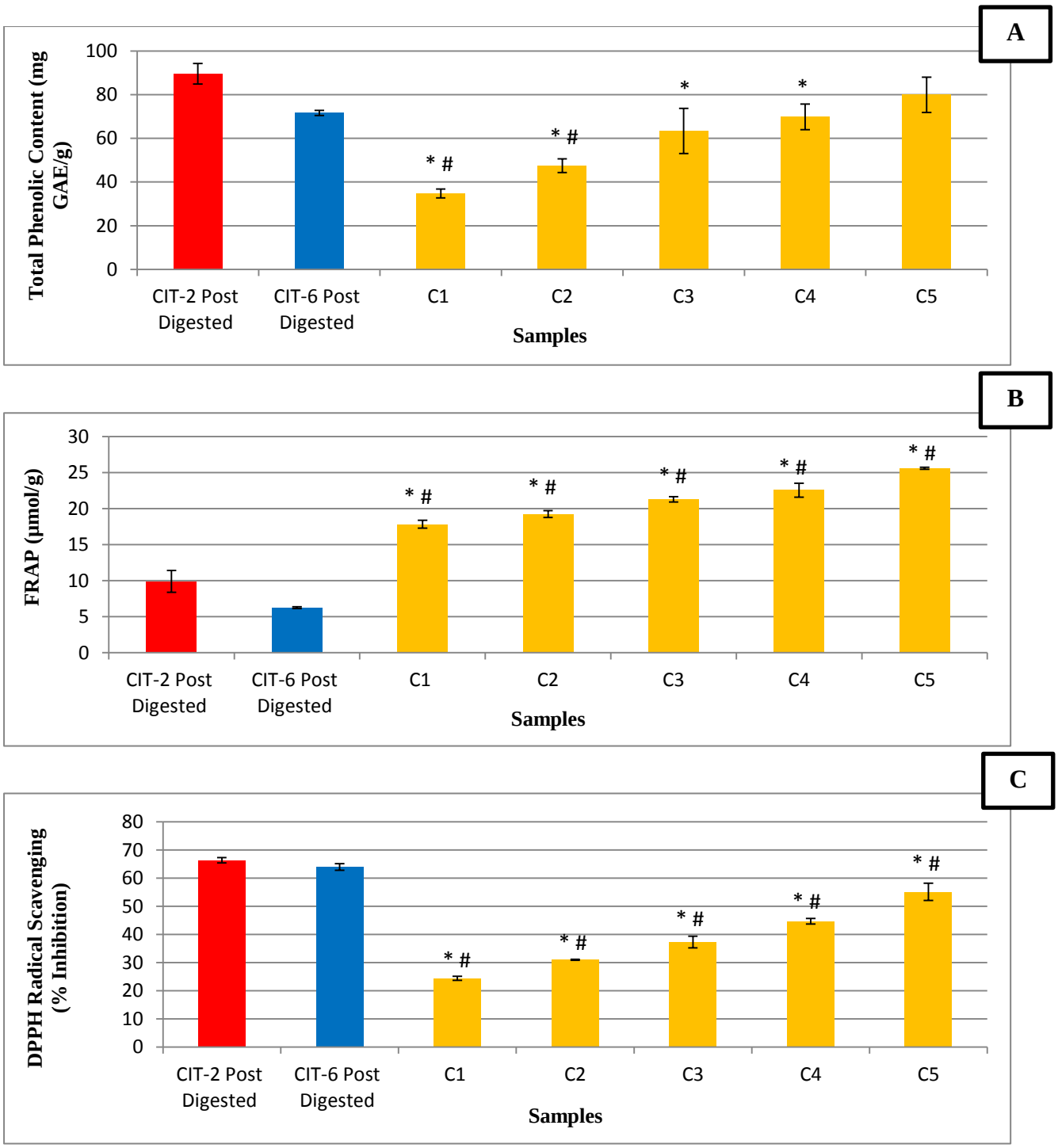

Figure 2.5: TPC/FCR Reducing Capacity (A); FRAP Activity (B); DPPH Scavenging Activity (C) of BSG CIT 2 & CIT 6 fractions post digestion vs. post digestion combinations. * denotes a statistically significant ($p<0.05$) difference between the protein digestate to protein & phenolic digestate combinations**.** # denotes a statistically significant ($p<0.05$) difference between the phenolic digestate to protein & phenolic digestate combinations. Data represents mean ± standard deviation from three independent experimental replicates.

Table 2.6: TPC, FRAP & DPPH Assay Results for digested CIT-2 & CIT-6 BSG-derived fractions and digestate combinations. Data represents mean ± standard deviation (SD) values based on three independent experiments. ***** denotes a statistically significant ($p<0.05$) difference between the protein digestate and the protein & phenolic digestate combinations**. #** denotes a statistically significant ($p<0.05$) difference between the phenolic digestate and the protein & phenolic digestate combinations.

Sample	**TPC Assay Average (mg GAE/g sample ± SD)**	**FRAP Assay Average (µmol/g sample ± SD)**	**DPPH Assay Average (% Inhibition ± SD)**
CIT-2	89.604 ± 4.72	9.898 ± 1.50	66.355 ± 0.95
CIT-6	71.660 ± 1.19	6.246 ± 0.13	63.993 ± 1.15
Combination 1 (C1) CIT-2 (10%) & CIT-6 (90%)	34.772 ± 1.98*#	17.836 ± 0.54*#	24.386 ± 0.72*#
Combination 2 (C2) CIT-2 (30%) & CIT-6 (70%)	47.477 ± 3.16*#	19.245 ± 0.49*#	30.969 ± 0.19*#
Combination 3 (C3) CIT-2 (50%) & CIT-6 (50%)	63.361 ± 10.34*	21.274 ± 0.37*#	37.319 ± 2.06*#
Combination 4 (C4) CIT-2 (70%) & CIT-6 (30%)	69.826 ± 5.90*	22.554 ± 0.98*#	44.644 ± 0.99*#
Combination 5 (C5) CIT-2 (90%) & CIT-6 (10%)	79.918 ± 8.08	25.614 ± 0.14*#	55.166 ± 3.05*#

2.4 Discussion

BSG is a significant waste product of the brewing industry (Guido & Moreira, 2017), and identifying alternative ways to use it would have both environmental and economic advantages (Bolwig et al., 2019). As BSG is a rich source of bioactive compounds (Almeida et al., 2017), one option is to incorporate BSG and/or its components as ingredients in healthy food products for older adults.

The older population has impaired antioxidant defence systems and elevated oxidative stress levels, which contribute to the ageing process but also promote the development of chronic age-related diseases (Goñi & Hernández-Galiot, 2019). Fusco et al. (2007) revealed that on a daily basis approximately 1% of reactive oxygen species play a part in the oxidative damage of tissues and subsequently contribute to the aging process by means of escaping endogenous anti-oxidant defences. A study conducted by Zujko et al. (2015) revealed that older adults, particularly women, have significantly low levels of antioxidants in their diet compared to younger adults. Designing foods that could increase dietary antioxidants is one strategy to try and promote healthy ageing.

The antioxidant potential of BSG has been extensively reported (Birsan et al., 2019; Almeida et al., 2017; Kitrytė et al., 2015; Connolly et al., 2013). Of interest, some studies have demonstrated that modifying the extraction methods used to isolate BSG-derived fractions can impact the recovery and stability of the inherent bioactive compounds (Bonifácio-Lopes et al., 2019; Guido & Moreira, 2017). Meneses et al. (2013) demonstrated that a solid-to-liquid extraction method using 60% v/v acetone:water mixture enabled a higher yield of antioxidant material. Modifying the enzymes used to hydrolyse BSG in the extraction procedures (Shen et al., 2019a; Treimo et al., 2008; Celus et al., 2007) and using different pre-treatment options and incubation conditions have also been shown to impact protein yield

(Qin et al., 2018). In the current study a variety of changes were made to the extraction methods to try and improve the yield of bioactive-rich material from hydrolysed BSG. While all fractions were associated with antioxidant properties, some fractions were more potent and associated with significantly higher antioxidant activity compared to intact BSG. The data demonstrates that the BSG-derived fractions had significantly different ($p<0.05$) antioxidant properties to each other and the intact BSG, which confirms that the extraction methods did influence bioactivity

CIT-15 was reported to be the least antioxidant BSG-derived fraction (Table 2.4 & Table 2.5). The most potent antioxidant fractions were identified as CIT-2, CIT-4, CIT-14 and CIT-17, which were all protein-derived hydrolysed fractions of BSG. Of these CIT-4 and CIT-14 were the most potent and these fractions were isolated following modifications to the enzyme reaction conditions and further fractionation, suggesting that the additional steps in the extraction procedure were warranted.

Two phenolic fractions were isolated (CIT-3 and CIT-6) following different enzyme extractions, and while both were associated with significantly higher ($p<0.05$) antioxidant properties compared to the intact BSG, the fraction generated by incubating with carbohydrases for a shorter incubation time (CIT-3) had higher antioxidant activity. Ultrafiltration of the phenolic fraction CIT-6 did not significantly improve the antioxidant properties of this fraction, indicating that further fractionation of the phenolic fraction was not beneficial.

The fibre sediment fractions were associated with antioxidant properties with CIT-16 overall demonstrating greatest activity in comparison to CIT-15, indicating the impact of alternative extraction methods. However, these fractions were not as bioactive as the protein and phenolic-rich fractions. The fat content of products has been shown to influence how compounds, such as flavonoids, phenolic acid and pro-anthocyanidin, are extracted

(Wojtunik-Kulesza et al., 2020). Data from the current study suggests that fat content can influence bioactivity but in this study defatting BSG-derived fractions CIT-2 and CIT-19 did not overall significantly improve the antioxidant properties of the fractions. Nevertheless, it was evident that CIT-17, which was generated from CIT-2, exhibited greater antioxidant activity than CIT-18, which was yielded from CIT-19, with the exception of the DPPH assay (Figure 2.1 and Table 2.4).

As can be seen in Table 2.4 and Table 2.5, following a multiple comparison test, the majority of BSG-derived fractions presented significantly different ($p<0.05$) antioxidant activities to each other. Interestingly, CIT-15 was the only fraction that was significantly different ($p<0.05$) to all BSG-derived fractions. However, this was only seen within the TPC assay and not the FRAP and DPPH assay (Table 2.4 and Table 2.5). The DPPH radical scavenging activity of BSG-derived fractions CIT-12 and CIT-17 proved to have no significance in comparison to each other or the other BSG-derived fractions DPPH radical scavenging activity (Table 2.4 and Table 2.5).

Digestion modifies biomolecules by means of degradation or transformation of the compounds into other structures that can have different chemical and biological properties (Wojtunik-Kulesza et al., 2020). It can also affect bioaccessibility and bioavailability of these compounds *in vivo* and, thus their potential beneficial bioactivities (Wojtunik-Kulesza et al., 2020). How the chemical and physical gastro-environment influences biological structures can vary, depending on the compound and the food matrix in which it is enclosed (Wojtunik - Kulesza et al., 2020; Buitimea-Cantúa et al., 2018; Chandrasekara & Shahidi, 2012). Several studies have reported on the changes of BSG polyphenol structures during digestion. For example, BSG contains flavon-3-ols and flavonoids (Martín-García et al., 2019; Stefanello et al., 2018; Almeida et al., 2017). Flavon-3-ols can survive the harsh acidic conditions of the stomach and can remain unaltered as they pass into the duodenum (Wojtunik-Kulesza et al.,

2020). Flavonoids undergo a variety of structural modifications (such as methylation, deglycosylation, hydroxylation, glucuronidation and sulphonation) within the small intestine due to the high pH (Wojtunik-Kulesza et al., 2020). These changes can influence their antioxidant properties. BSG proteins are a source of bioactive peptides, which are released when digested by proteases (Shen et al., 2019a; Mazorra-Manzano et al., 2017; Treimo et al., 2008).

In vitro digestion models can provide relevant biological data on food-derived compounds and help elucidate how digestion impacts their bioactivity. The INFOGEST static *in vitro* gastrointestinal model was used in this study to investigate the antioxidant properties of the BSG-derived fractions following digestion. While it is important to acknowledge that *in vitro* conditions do not completely mimic *in vivo* conditions, it is reasonable to suggest that data for *in vitro* BSG digested fractions is more physiologically relevant than undigested fractions. Data from this study demonstrated that digestion did impact the antioxidant properties of BSG-derived fractions. These results agree with other studies that investigated the impact of *in vitro* digestion on the antioxidant properties of grains. Chandrasekara & Shahidi (2012) reported that the *in vitro* digestion impacted the antioxidant potential of phenolic compounds in dehulled millet grains. In addition, Gong et al. (2013) revealed that the total antioxidant capacity of digested whole-grain cereals (rice, wheat, buckwheat, barley) were lower than their undigested equivalents. Zhang et al. (2020) reported a similar pattern in the DPPH radical scavenging power and TPC of two different varieties of wheat and green wheat following *in vitro* digestion. Pavan et al. (2014) also revealed that the TPC of both papaya and araticum decreased following *in vitro* digestion, while the TPC of the jackfruit increased.

Combining food compounds can potentially have synergistic effects and enhance the bioactive properties of the combination. Studies have reported synergy between protein and phenolic compounds. Dai et al. (2019) discovered that the antioxidant capacity of a rice

polyphenol, B-type procyanidin dimer is protected and improved when combined with a rice protein, glutelin. The authors suggested that the interaction between the protein and phenolic compounds lowered the risk of the polyphenolic compounds interacting with environmental pro-oxidants (Dai et al., 2019). Protein-phenolic compound interactions can prevent degradation of the phenolic compounds during digestion, with the protein functioning as a protective carrier for the phenolic compound (Buitimea-Cantúa et al., 2018). In this study, combining the protein and phenolic BSG fractions did not overall improve their antioxidant properties, in fact antagonistic effects were largely observed. This may be due to the fact that the fractions were digested first before they were combined, thus hydrolysing proteins and possibly preventing protein-phenolic interactions, thus eliminating any protective effects from the proteins.

It is important to acknowledge that while *in vitro* chemical-based assays are economical and suitable high-throughput screening assays (López-Alarcón & Denicola, 2013), additional *in vitro* studies using cell-based models or *in vivo* animal models would further confirm the improved antioxidant properties of some of the novel BSG fractions.

2.5 Conclusion

BSG is an antioxidant-rich substance and has potential as an ingredient in functional foods for older adults. In this study the bioactivity of this abundant agro-industry by-product was improved by modulating the extraction procedures to produce more potent antioxidant fractions. While all BSG-derived fractions were associated with antioxidant properties, in particular, isolating peptide-rich fractions and defatting protein hydrolysates significantly improved the antioxidant potential of BSG. Further studies of interest would be to investigate if other bioactivities were also impacted/improved by the changes/modifications to the extraction procedures. Combining fractions did not appear to improve antioxidant properties, however, only a select number of fractions were investigated, thus it may be of interest to combine other fractions to determine if synergistic effects are observed. In addition, combining the fractions first, before digestion could improve activity, but this would need to be investigated. This study also demonstrated that digestion impacted the bioactive properties of BSG-derived fractions, providing more physiologically relevant data regarding the potential bioaccessibility of antioxidant compounds. Data from this study suggests that the bioactive properties of BSG-derived fractions can be improved by altering extraction methods. BSG-derived fractions with antioxidant properties could be incorporated into foods targeted at older adults in order to increase their antioxidant intake and support healthy aging. However, as this data was based on *in vitro* chemical-based assays, it would be of interest to confirm the antioxidant properties of the BSG-derived fractions using *in vitro* cell-models initially, and subsequently *in vivo* animal models.

Chapter 3

Cellular-based Antioxidant & Immunomodulatory Properties of BSG-Derived fractions

3.0 Abstract

Brewers' spent grain is an inexpensive brewing industry co-product that is associated with several health promoting properties. In particular, the antioxidant properties of BSG have been extensively reported. While BSG is primarily used as animal feed there is now significant interest in identifying alternative applications for BSG. One strategy is to isolate potent bioactive BSG-derived fractions that could be used as ingredients to fortify human food products. This study investigated the cellular antioxidant and anti-inflammatory potential of 20 BSG-derived fractions that were digested using a simulated *in vitro* gastrointestinal digestion (SIGD) model. Cytotoxicity was initially assessed using the MTT assay. Cellular antioxidant investigations were performed using the human monocytic cell line, U937, and the superoxide dismutase (SOD) assay. The immunomodulatory properties of the BSG-derived fractions was investigated by measuring cytokine production (TNF-α) in concanavalin A (conA) stimulated Jurkat T cells. Two fractions demonstrated significantly higher ($p<0.05$) antioxidant potential compared to intact BSG. A peptide-rich, BSG-derived fraction displayed the greatest cellular antioxidant potential and was also associated with immunomodulatory properties. All fractions reduced TNF-α secretion *in vitro* at the concentrations tested but none of the fractions were significantly different to the positive control. Fractionating BSG can generate more potent bioactive fractions but studies are needed to further elucidate their immunomodulatory mechanisms and improve their potential as bioactive ingredients in functional foods.

3.1 Introduction

Every year 8.5 million tons of BSG is generated globally (McCarthy et al., 2013b). BSG is an inexpensive, heterogeneous by-product of the brewing industry, and primarily consists of protein (19-30%), fibre (30-50%) and lignin (10-28%) (Lynch et al, 2016). The components of BSG, particularly the protein, fibre and phenolic compounds, are associated with a variety of health-promoting properties including prebiotic (Lao et al., 2020; Sajib et al., 2018; Gómez et al., 2015; Reis et al., 2014b), anticancer (Ikram et al., 2017), antioxidant (Wen et al., 2019; Vieira et al., 2017; Crowley et al., 2017; Moreira et al., 2013) and immunomodulatory properties (Cian et al., 2020; Wen et al., 2019; McCarthy et al., 2015; Crowley et al., 2015; McCarthy et al., 2013a; McCarthy et al., 2013b; McCarthy et al., 2013c). Several groups have reported on the potential gut-health properties of BSG, with particular focus on its prebiotic properties (Lao et al., 2020; Gomez et al., 2015). In 2012 McCarthy et al. demonstrated the cellular antioxidant potential of BSG polyphenols *in vitro* using the lymphocytic U937 cell line (McCarthy et al., 2012) and the same group also reported on the potential immunomodulatory properties of BSG (McCarthy et al. 2013a; McCarthy et al. 2013b). These associated health-promoting properties have prompted alternative uses for BSG, particularly as an ingredient in foods for human consumption. BSG has been added to several food products to date including baked goods (Lukinac et al., 2015; Plessas et al., 2007; Hassona, 1993), ready-to-eat snacks (Steinmacher et al., 2012) and dairy products (Crowley et al., 2015). However, additional studies are needed to confirm if adding grains like BSG to foods have the expected *in vivo* health benefits.

A study by Kopf et al. (2018) demonstrated that incorporating whole grain foods in the diet can positively influence gut microbiota and inflammatory cytokines in obese/overweight subjects, thus decreasing the risk of subclinical inflammation and improving metabolic

health. In addition, modifying extraction methods allows more potent bioactive fractions to be isolated, which could be subsequently added to foods to further help design novel functional foods and potentially improve their health benefits. McCarthy et al. (2013a) demonstrated that changing the proteases used to hydrolyse BSG modified the peptide profile, whereby flavourzyme generated hydrolysates containing less peptides < 5kDa compared to enzymatic hydrolysis with corolase and alcalase. This can potentially influence the bioactive properties of BSG extracts (McCarthy et al., 2013a). Modifying physical and thermal pre-treatment methods can also alter composition of BSG extracts, potentially increasing the yield and potency of the bioactive material (Ravindran et al., 2018; Lynch et al., 2016; Niemi et al., 2013b; Niemi et al., 2012a).

Cellular antioxidant assays primarily help to detect the activity of exogenous antioxidant compounds in food, thus highlighting their ability to decrease or inhibit oxidative stress (McCarthy et al., 2013b). Superoxide dismutase (SOD) is one of three principle enzymatic-endogenous antioxidants that under aerobic conditions convert reactive oxygen species and their by-products into less reactive forms, thus protecting the body from damage caused by oxidative stress and inflammation (Younus, 2018; Asakura & Kitahora, 2018; Singh et al., 2017; Ighodaro & Akinloye, 2017). SOD levels have been shown to decline with age (Ighodaro & Akinloye, 2017) and reduced or imbalanced SOD activity has been correlated with health problems (Quaye et al., 2019; Younus, 2018; Gheddouchi et al., 2015; Elmasry et al., 2015; Feoli et al., 2014; Ahmad et al., 2012). The SOD assay was used to investigate the antioxidant potential of BSG-derived fractions. Due to the fact that BSG is now recognised as a nutritionally valuable product, there is currently a lot of interest in using it to formulate health-promoting functional foods (Fărcaş et al., 2014b).

The superoxide dismutase (SOD) assay involves WST-1 (water soluble tetrazolium) and xanthine oxidase and is a simple and ideal method to analyse large quantities of samples (Peskin & Winterbourn, 2017). The presence of a superoxide radical generates a yellow coloured formazan product (WST-1 formazan) which can be quantified spectrophotometrically. SOD is an antioxidant enzyme that can reduce the free radical and therefore the amount of formazan produced, which is reflected by a decrease in absorbance (Sigma-Aldrich, 2018; Peskin & Winterbourn. 2017). In the presence of another antioxidant, there is competition between this antioxidant and SOD to reduce the free radical. Test samples with high antioxidant activity result in less formazan product and thus, a lower absorbance value is measured. Absorbance is inversely proportional to the inhibition of SOD (Sigma-Aldrich, 2018).

The potential immunomodulatory properties of foods and food-derived compounds can be investigated *in vitro* using cell-based assays. The general principle of these assays is based on measuring the release of specific immune markers (e.g. cytokines) from an appropriate cell model that has been stimulated by an inflammatory agent. One of the most frequently measured immune markers is tumor necrosis factor alpha (TNFα), a 26-kD cytokine protein and major immune regulator that has both anti-inflammatory and pro-inflammatory properties and plays a vital function in cellular immune response (Ravussin & Smith, 2016; Sedger & McDermott, 2014; Parameswaran & Patial, 2010; Wang & Lin, 2008; Moss et al., 1997). *In vivo,* it is secreted predominantly by activated macrophages and monocytes (Ravussin & Smith, 2016; Sellati & Sahay, 2014; Moss et al., 1997). TNFα works through two receptors known as TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) (Ye et al., 2018; Parameswaran & Patial, 2010). TNFα assembles and binds large quantities of high-affinity receptors on cell membranes in order to exert its effects through secondary protein activation (e.g. mitochondrial proteins, GProteins, phospholipases and transcription factors), which

further triggers cellular responses such as nitrogen/reactive oxygen radical generation and/or gene transcription (Idriss & Naismith, 2000). TNFα is involved in cell survival by means of NF-κB activation as well as apoptotic or necrotic cell death, with TNFα deficiencies leading to progressive disease development and potentially death (Idriss & Naismith, 2000). Within an appropriate cell model, if levels of TNFα are modulated in response to test compounds it indicates they have potential immunomodulatory properties.

One of the most frequently used methods to measure levels of cytokines, such as TNFα, is enzyme-linked immunosorption assays (ELISA). These assays use specific antibodies to form immunocomplexes with targeted antigenic immune proteins and are highly sensitive and specific assays that are suitable for qualitative and quantitative analysis (Sakamoto et al., 2018). Antigens are quantified between antibody layers (Sakamoto et al., 2018; Burguillos, 2013) that include a capture antibody coated onto a 96-well microplate, which binds to any antigen in the test sample. Then a detection antibody binds to the immunocomplex that has formed. A streptavidin protein that is enzyme labelled [horseradish peroxidase (HRP) enzyme] is then added, which binds to a nonspecific region of the detection antibody, followed by the addition of a suitable substrate which reacts with the enzyme and a colour develops that is quantified, usually spectrophotometrically (R&D Systems, 2019).

In the present study the antioxidant and anti-inflammatory properties of novel BSG-derived fractions was investigated using cell-based *in vitro* models. These *in vitro* models have more physiological relevance to what could happen *in vivo* and are utilised to investigate and examine cell behaviour, cell biochemistry and physiology, within a regulated environment, (Hudu et al., 2016; Amorati & Valgimigli, 2015; Arango et al., 2013). To generate the novel fractions a variety of changes were made to the enzymatic hydrolysis process and these mainly included altering the types of enzymes used, the pH, incubation conditions and the

fractionation steps. The cytotoxicity effects of the novel BSG-derived fractions were initially investigated in both the human monocytic blood cell lines (U937) and human leukaemic T cells (Jurkat T cells). BSG-derived fractions with improved antioxidant and/or anti-inflammatory properties would further the application of BSG and facilitate the development of novel functional foods.

3.2 Materials and Methods

3.2.1 Materials

Reagents sourced from Sigma-Aldrich: RPMI-1640 Medium, Penicillin-Streptomycin (Pen-Strep), Phosphate Buffered Saline (PBS), Thiazolyl Blue Tetrazolium Bromide (MTT), Superoxide Dismutase (SOD) Determination Kit and Concanavalin A from *Canavalia ensiformis*, Jack bean (ConA). **Reagents sourced from ThermoFisher Scientific:** Gibco Fetal Bovine Serum (FBS). **Reagents sourced from R&D Systems Inc.**: DY210 - Human TNF-alpha DuoSet ELISA, DY008 - DuoSet ELISA Ancillary Reagent Kit 2

3.2.2 BSG Sample Preparation

BSG (supplied by Diageo) was processed and fractionated according to the methods described in Section 2.2.2, generating 20 BSG derived fractions (CIT-2–CIT-21) (Table 2.1). These included a protein-rich hydrolysate fraction (CIT-2) and a phenolic-rich fraction (CIT-6), as well as 18 novel BSG-derived fractions produced using a variety of different extraction methods. These fractions were digested as outlined in section 2.2.3 and the digestates were further investigated to examine their potential cellular antioxidant and anti-inflammatory properties.

3.2.3 Cell Culture

The human monocytic blood cell line, U937 (-ATCC®, #CRL-1593.2™) was utilized for cellular antioxidant investigations while the human leukaemic T cell line, Jurkat T (- ATCC®, #TIB-152™) was used for immunomodulatory investigations. Both cell lines were cultured and maintained according to supplier guidelines and investigated using methods described by Crowley et al. (2017), McCarthy et al. (2015), Crowley et al. (2015) and McCarthy et al. (2013a). Cells were cultured in RPMI-1640 medium supplemented with 10% v/v foetal

bovine serum (FBS) and 1% v/v Penicillin-Streptomycin (Pen-Strep). For experiments, U937 cells and Jurkat T cells were used between passage numbers 15 and 35.

3.2.4 Cell Proliferation

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to measure cellular viability and identify sub-toxic concentrations of BSG fractions for cell culture experiments (McCarthy et al., 2013a). For this, cells were adjusted to a density of $1x10^6$ cells per ml in antibiotic-free medium, supplemented with 2.5% foetal bovine serum (FBS) and then 50µl of cell suspension was seeded into 96-well plates and incubated for 24hrs at 37°C, 5% CO_2. Following the incubation period, 50µl of BSG digestate fractions were added at concentrations of 0, 0.5, 1, 2, 2.5, 5 & 10% (v/v) in antibiotic-free medium, supplemented with 2.5% foetal bovine serum (FBS) to bring the final volume to 100µl and the plates were then incubated for a further 24hrs at 37°C. After 24hrs, 10µL of MTT reagent (5mg/1ml PBS) was added to each well and the plate was wrapped with foil and incubated for a 4hr period at 37°C, 5% CO_2. 100 µL of solubilisation solution (6mls of 2% Glacial Acetic Acid + 4mls of DMF + 1.6g SDS) was then added to each well and absorbance was measured spectrophotometrically using a microplate reader (Varioskan™ LUX multimode microplate reader) at 570nm (test wavelength) and 630nm (reference wavelength). A media blank (no cells) was used to account for the background colour of the BSG digestate fractions.

3.2.5 Antioxidant Investigations

The antioxidant potential of the BSG-derived digested fractions was assessed using a cell-based superoxide dismutase (SOD) assay in accordance with the manufacturer's guidelines and adapted according to McCarthy et al. (2013a; 2013b; 2013c). The principle of this assay is based on the competition between the xanthine–xanthine oxidase system which generates

the superoxide radical (O_2^-) and the activity of the SOD enzyme to catalyse the breakdown of O_2^- to oxygen (O_2) and hydrogen peroxide (H_2O_2) (Čolak & Žorić, 2019), thus reducing a water-soluble tetrazolium salt, i.e., 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt (WST-1) to the corresponding formazan (WST-1formazan) absorbing at 450nm (Figure 3.1). Therefore, absorbance at 450nm in this indirect colorimetric method is proportional to O_2^- concentration, thus reduction in colour development quantifies SOD activity as an inhibition activity. One unit of SOD activity is the total sample enzyme required for a tetrazolium salt reduction by 50% inhibition (IC50) under specified conditions (Zhou & Prognon, 2006).

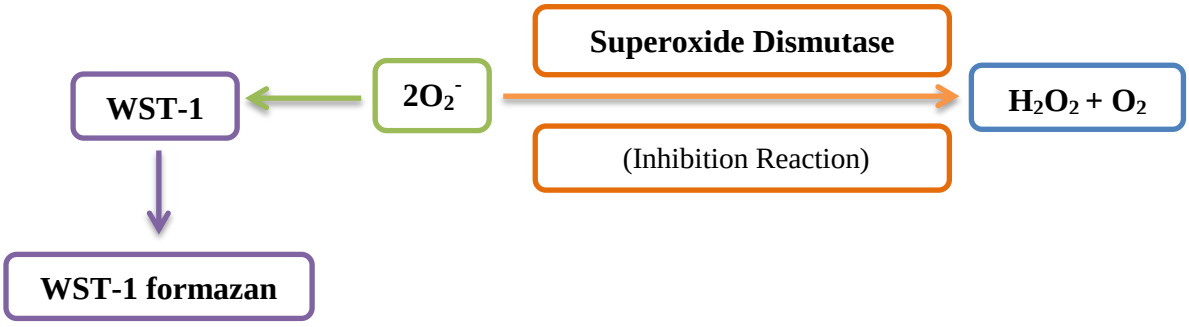

Figure 3.1: SOD Assay Principle

For each experiment, 5mL of 1×10^5 U937 cells per mL were seeded into a T25 flask (Sarstedt) in antibiotic-free (RPMI) media, supplemented with 5% FBS and incubated for 24hrs at 37°C, 5% CO_2 with the selected non-toxic concentration of BSG digestate fraction. Following incubation, cells were harvested, sonicated (MSE Soniprep 150 Ultrasonic disintegrator) on ice (3 times at 10z) and centrifuged at 72 RCF/G-force for 10mins (Jouan C 412 Centrifuge) before the supernatant was removed for analysis.

To assess the antioxidant activity, reactions were set up according to manufactures instructions (SIGMA: 19160 SOD Determination Kit) and (Table 3.1) and incubated for

20mins at 37°C, 5% CO_2. The absorbance was measured at 450nm using a microplate reader (Varioskan™ LUX multimode microplate reader). Absorbance is inversely proportional to the SOD antioxidant enzyme activity (% Inhibition Rate). The SOD activity was determined using the following equation:

(% Inhibition Rate) = {[(S1 - S3) - (SS - S2)] / (S1 – S3)} x 100%.

Sample (BSG-derived samples CIT 1 – CIT 21)

Blank 1 (Negative control, with enzyme)

Blank 2 (Negative sample control, without enzyme)

Blank 3 (Negative control, without enzyme)

Table 3.1A: Preparation of SOD Assay

	Sample	Blank 1	Blank 2	Blank3
Sample	**20µl**	**-**	**20µl**	**-**
Deionised H_2O	**-**	**20µl**	**-**	**20µl**
WST Working solution (WST) (1:20)	**200µl**	**200µl**	**200µl**	**200µl**
Enzyme Working Solution	**20µl**	**20µl**	**-**	**-**
Dilution Buffer	**-**	**-**	**20µl**	**20µl**

3.2.6 Immunomodulatory Investigations

To assess the immunomodulatory properties of the BSG-derived fractions, 9mls of 2×10^5 cells per mL suspensions in antibiotic-free (RPMI) media, supplemented with 5% FBS, were seeded into T25 flasks (Sarstedt). These cells were treated with 90µl of the pro-inflammatory agent Concanavalin-A (50mg/ml^{-1}) and incubated for 24hrs at 37°C, 5% CO_2. Following this, cell suspensions (100µL) were seeded, using an appropriate number of test wells, into 96 well plates. Sample test wells contained 40µL of a 2% sample. The three

control wells were set up according to Table 3.1B. Plates were then incubated for a further 24hrs at 37°C, 5% CO_2. Following incubation, plates were centrifuged for 5mins (Jouan C 412 Centrifuge) and all supernatants were collected in sterile eppendorf tubes and stored in the -20°C freezer until further analysed. All samples were analysed in duplicate.

Table 3.1B: ELISA Assay – Setup

Components	Control 1	Control 2	Control 3	BSG Sample
Negative Control 1 Media (5% FBS, RPMI)	**200µl**	**100µl**	**100µl**	**60µl**
Negative Control 2 Jurkat T Cells	-	**100µl**	-	-
Positive Control Jurkat T cells & Con A	-	-	**100µl**	**100µl**
Sample (2%)	-	-	-	**40µl**

TNFα secretion was measured using a Human TNF-alpha DuoSet ELISA kit **(**R&D Systems, Inc.). The assay was set up in accordance with manufacturer's instructions and the sample absorbance was measured at 450nm and 570nm using a microplate reader (Varioskan™ LUX multimode microplate reader). For wavelength correction absorbance, readings at 570nm were subtracted from absorbance readings at 450nm. Data was analysed using online ELISA Analysis Software (elisaanalysis.com).

3.2.7 Statistical Analysis

The bioactivities of each BSG-derived fraction following *in vitro* digestion were analysed in three independent experiments. Data presented represents the average ± standard deviation of all measurements. Statistical analysis was carried out using the IBM Statistical Package for the Social Sciences (SPSS v.26). A one-way analysis of variance (ANOVA) was used to compare differences in the bioactivity between all BSG digestates. Controlling for multiple comparisons, the Dunnett's post-hoc test was used to evaluate mean changes between

digestates and a control sample. A p-value <0.05 was considered statistically significant (Appendices, Pages 212-213; 214-215).

3.3 Results

3.3.1 Cell Cytotoxicity

The BSG-derived digestate fractions were screened for their cytotoxic effects on both the U937 and Jurkat T cell lines using the MTT assay. Data indicated that the fractions reduced cell proliferation in U937 and Jurkat T cells, however not in a dose-dependent manner for all fractions (Tables 3.2A and Table 3.2B). Indeed, certain samples appeared to stimulate growth. A concentration of 1% (v/v) was chosen for analysis of potential antioxidant activity in U937 cells and a concentration of 2% (v/v) was chosen for immunomodulatory investigations. These concentrations were based on the effects of the BSG digestates on cell proliferation and a cut-off of > 80% (for 95% of the test fractions) (Table 3.2A; Table 3.2B).

Table 3.2A: Effect of novel BSG-derived fractions (0-5%) on cell proliferation (% of control) in the U937 cell line following *in vitro* digestion

Sample	CIT Code	0%	0.5%	1%	2%	2.5%	5%
BSG Intact	CIT-1	100.00	96.84	83.51	87.11	83.13	62.04
Protein Hydrolysate	CIT-2	100.00	119.00	120.21	119.33	114.60	131.30
New Phenolic Extraction	CIT-3	100.00	83.12	81.03	80.12	80.76	70.76
Soluble Fraction	CIT-4	100.00	52.44	92.27	106.02	48.14	85.04
Insoluble Fraction	CIT-5	100.00	92.23	81.87	80.11	67.75	10.91
Phenolic Rich Fraction	CIT-6	100.00	57.12	29.01	4.58	2.58	0.33
Permeate 3kDa	CIT-7	100.00	91.19	81.86	71.50	63.06	4.68
Permeate 10kDa	CIT-8	100.00	87.88	78.74	70.73	62.10	15.76
Permeate 50kDa	CIT-9	100.00	90.43	85.95	69.71	66.28	3.48
Not Separated Fraction	CIT-10	100.00	122.21	107.33	141.27	115.28	66.83
Retentate 3kDa	CIT-11	100.00	100.99	97.79	95.80	94.41	28.43
Retentate 10kDa	CIT-12	100.00	96.56	90.52	79.25	69.46	0.99
Retentate 50kDa	CIT-13	100.00	90.47	72.97	61.64	47.89	5.08
BSG Peptide Rich Fraction	CIT-14	100.00	97.85	105.66	99.73	176.65	
Old Fibre Rich Fraction	CIT-15	100.00	86.270	87.79	78.36	61.86	1.70
New Fibre Rich Fraction	CIT-16	100.00	104.19	101.08	75.74	99.28	43.94
Defatted Hydrolysate (d1)	CIT-17	100.00	107.06	93.19	80.97	77.07	16.51
Defatted Hydrolysate (d2)	CIT-18	100.00	95.98	87.06	79.56	71.53	15.85
Hydrolysate da	CIT-19	100.00	107.81	103.57	86.93	45.88	4.89
H20 Extraction	CIT-20	100.00	93.40	89.63	88.25	83.39	35.46
BSG Aqueous Extraction	CIT-21	100.00	128.72	108.16	92.84	83.22	20.71

Table 3.2B: Effect of novel BSG-derived fractions (0-5%) on cell proliferation (% of control) in the Jurkat cell line following *in vitro* digestion

Sample	CIT Code	0%	0.5%	1%	2%	2.5%	5%
BSG Intact	CIT-1	100.00	105.41	103.80	92.62	84.84	57.12
Protein Hydrolysate	CIT-2	100.00	85.58	90.37	83.32	79.03	50.06
New Phenolic Extraction	CIT-3	100.00	130.61	130.82	125.44	114.48	48.89
Soluble Fraction	CIT-4	100.00	96.21	99.78	100.68	122.41	99.80
Insoluble Fraction	CIT-5	100.00	101.06	102.23	87.78	77.44	49.80
Phenolic Rich Fraction	CIT-6	100.00	94.62	87.88	81.07	77.34	50.02
Permeate 3kDa	CIT-7	100.00	93.05	82.76	83.01	72.85	37.34
Permeate 10kDa	CIT-8	100.00	101.78	101.75	93.02	80.14	49.52
Permeate 50kDa	CIT-9	100.00	107.35	102.04	91.43	82.18	56.88
Not Separated Fraction	CIT-10	100.00	194.58	105.85	298.80	65.49	52.33
Retentate 3kDa	CIT-11	100.00	92.73	96.08	108.69	108.62	55.73
Retentate 10kDa	CIT-12	100.00	107.22	108.74	84.62	71.80	48.48
Retentate 50kDa	CIT-13	100.00	93.23	91.86	79.03	67.36	24.26
BSG Peptide Rich Fraction	CIT-14	100.00	79.46	75.14	93.95	72.23	117.61
Old Fibre Rich Fraction	CIT-15	100.00	97.90	93.25	86.41	75.55	43.17
New Fibre Rich Fraction	CIT-16	100.00	96.99	98.96	91.41	79.01	49.30
Defatted Hydrolysate (d1)	CIT-17	100.00	102.51	111.87	101.42	91.15	13.25
Defatted Hydrolysate (d2)	CIT-18	100.00	96.02	93.59	89.60	96.29	79.97
Hydrolysate da	CIT-19	100.00	71.41	82.45	86.38	84.83	53.12
H20 Extraction	CIT-20	100.00	96.11	101.09	93.40	88.93	60.59
BSG Aqueous Extraction	CIT-21	100.00	94.11	94.89	82.36	76.93	45.62

3.3.2 *Antioxidant effects of BSG-derived fractions*

Initially the cellular antioxidant activity of test samples CIT-1 (BSG intact fraction), CIT-2 (protein hydrolysate fraction) and CIT-6 (phenolic rich fraction) were compared. While CIT-2 demonstrated the highest cellular antioxidant activity (Figure 3.2A), there was no statistically significant difference between the three BSG-derived fractions.

Comparing the antioxidant properties of the novel BSG-derived fractions, data for SOD concentrations is presented in Table 3.3 and as can be seen values ranged from 20.050 ± 4.68 and 34.019 ± 4.61 U/mol. BSG Fractions CIT-20 (34.019 ± 4.61 U/mol), CIT-11 (33.819 ±

9.30 U/mol) and CIT-14 (31.139 ± 3.47 U/mol) showed the greatest cellular antioxidant potential while BSG fractions CIT-9 (20.050 ± 4.68 U/mol), CIT-5 (20.622 ± 4.26 U/mol) and CIT-7 (20.960 ± 3.99 U/mol) proved to have the weakest cellular antioxidant potential. When compared to the negative control (U937 cells only) none of the test fractions significantly increased SOD % Inhibition. However, CIT-14 and CIT-20 showed a significantly greater ($p<0.05$) antioxidant activity compared to the BSG intact fraction (CIT-1). As can be seen in Figure 3.2A and 3.2B, none of the BSG-derived fractions had significantly improved antioxidant properties compared to CIT-2 and CIT-6.

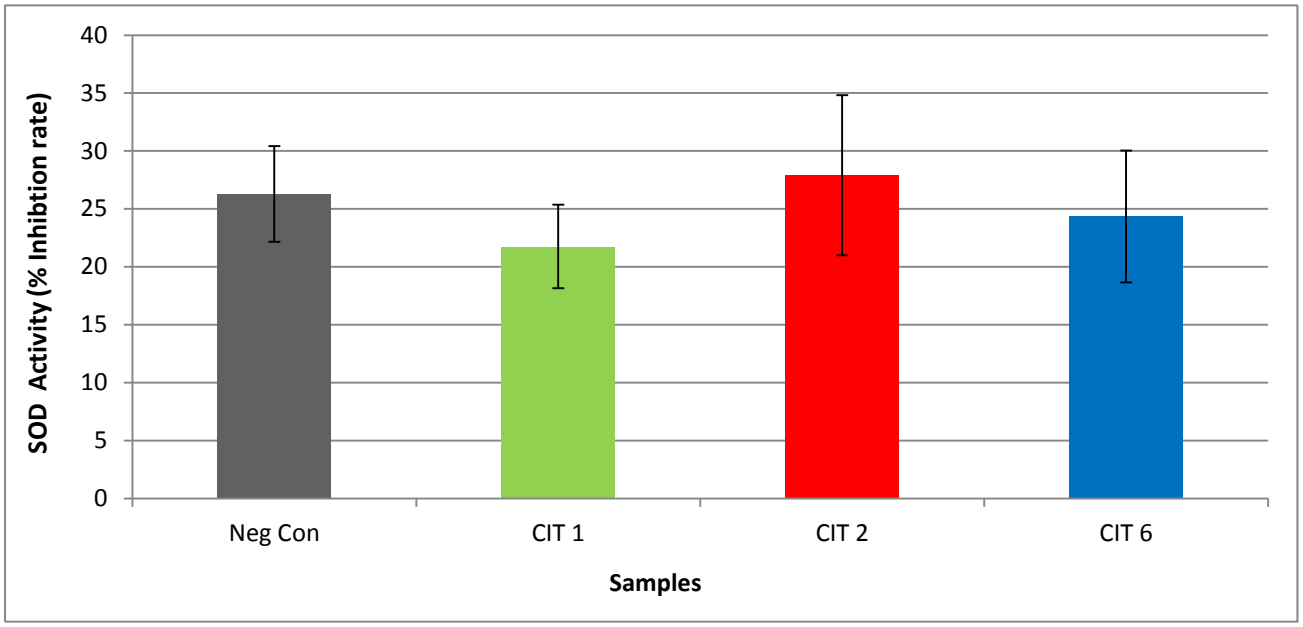

Figure 3.2A: SOD activity (% inhibition rate) of BSG-derived fractions, CIT-1 vs. CIT-2 and CIT-6 following *in vitro* digestion. No significant differences were observed between BSG-derived fractions and CIT-2 (protein) or between BSG-derived fractions and CIT-6 (phenolic).Data represents mean ± standard deviation from three independent experimental replicates.

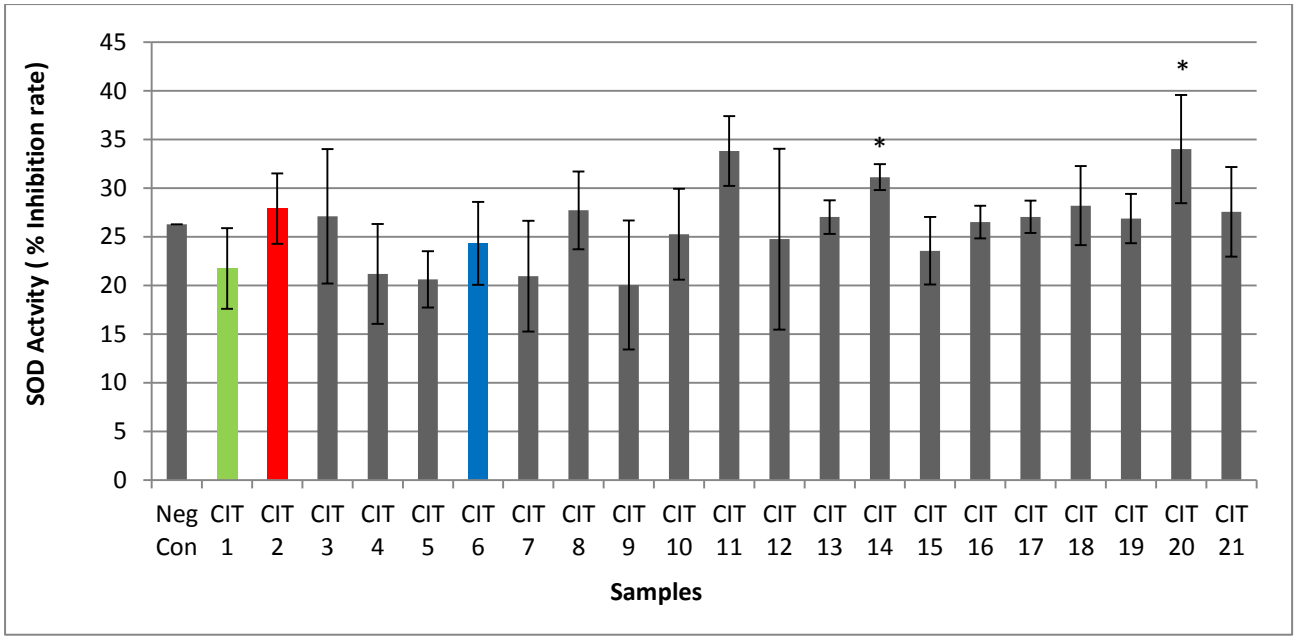

Figure 3.2B: SOD activity (% inhibition rate) of BSG-derived fractions (CIT-1-CIT-21) following *in vitro* digestion. ***** Denotes a statistically significant ($p<0.05$) difference between CIT-1 (BSG intact) and BSG digestate fractions. Data represents mean ± standard deviation from three independent experimental replicates.

Table 3.3: SOD Assay Results for BSG-derived fractions (CIT-1-CIT-21) following *in vitro* digestion. Data represents mean ± standard deviation (SD) values based on three independent experiments. * Denotes a statistically significant ($p<0.05$) difference between BSG fractions and the intact BSG (CIT-1)/Sample Control.

CIT Code	Sample	Average SOD Activity (% inhibition rate ± SD)
-	Negative Control	26.290 ± 4.14
CIT-1	BSG Intact	21.752 ± 3.61
CIT-2	Protein Hydrolysate	27.909 ± 6.90
CIT-3	New Phenolic Extraction	27.108 ± 5.13
CIT-4	Soluble Fraction	21.188 ± 2.90
CIT-5	Insoluble Fraction	20.622 ± 4.26
CIT-6	Phenolic Rich Fraction	24.334 ± 5.69
CIT-7	Permeate 3kDa	20.960 ± 3.99
CIT-8	Permeate 10kDa	27.722 ± 6.63
CIT-9	Permeate 50kDa	20.050 ± 4.68
CIT-10	Not Separated Fraction	25.273 ± 3.59
CIT-11	Retentate 3kDa	33.819 ± 9.30
CIT-12	Retentate 10kDa	24.773 ± 1.72
CIT-13	Retentate 50kDa	27.030 ± 1.32
CIT-14	BSG Peptide Rich Fraction	31.139 ± 3.47*
CIT-15	Old Fibre Rich Fraction	23.572 ± 1.67
CIT-16	New Fibre Rich Fraction	26.524 ± 1.66
CIT-17	Defatted Hydrolysate (d1)	27.062 ± 4.06
CIT-18	Defatted Hydrolysate (d2)	28.207 ± 2.53
CIT-19	Hydrolysate da	26.897 ± 5.57
CIT-20	H20 Extraction	34.019 ± 4.61*
CIT-21	BSG Aqueous Extraction	27.586 ± 3.13

Table 3.4: SOD Assay Results comparing the activity of all BSG-derived fractions relative to each other (CIT-2-CIT-21)/Multiple Comparisons following *in vitro* digestion. Data represents mean ± standard deviation (SD) values based on three independent experiments. Small superscript letters (a-t) denote a significant difference ($p<0.05$) in antioxidant activity, with the following associations: **CIT-2**=a; **CIT-3**=b; **CIT-4**=c; **CIT-5**=d; **CIT-6**=e; **CIT-7**=f; **CIT-8**=g; **CIT-9**=h; **CIT-10**=i; **CIT-11**=j; **CIT-12**=k; **CIT-13**=l; **CIT-14**=m; **CIT-15**=n; **CIT-16**=o; **CIT-17**=p; **CIT-18**=q; **CIT-19**=r; **CIT-20**=s; **CIT-21**=t, respectively

CIT Code	Sample	Average SOD (% Inhibition Rate ± SD)
CIT-2	Protein Hydrolysate	27.909 ± 6.90
CIT-3	New Phenolic Extraction	27.108 ± 5.13
CIT-4	Soluble Fraction	21.188 ± 2.90^{lms}
CIT-5	Insoluble Fraction	20.622 ± 4.26^{ms}
CIT-6	Phenolic Rich Fraction	24.334 ± 5.69
CIT-7	Permeate 3kDa	20.960 ± 3.99^{ms}
CIT-8	Permeate 10kDa	27.722 ± 6.63
CIT-9	Permeate 50kDa	20.050 ± 4.68^{ms}
CIT-10	Not Separated Fraction	25.273 ± 3.59
CIT-11	Retentate 3kDa	33.819 ± 9.30
CIT-12	Retentate 10kDa	24.773 ± 1.72^{ms}
CIT-13	Retentate 50kDa	27.030 ± 1.32^{cn}
CIT-14	BSG Peptide Rich Fraction	31.139 ± 3.47^{cdfhkn}
CIT-15	Old Fibre Rich Fraction	23.572 ± 1.67^{lms}
CIT-16	New Fibre Rich Fraction	26.524 ± 1.66
CIT-17	Defatted Hydrolysate (d1)	27.062 ± 4.06
CIT-18	Defatted Hydrolysate (d2)	28.207 ± 2.53^{c}
CIT-19	Hydrolysate da	26.897 ± 5.57
CIT-20	H20 Extraction	34.019 ± 4.61^{cdfhkn}
CIT-21	BSG Aqueous Extraction	27.586 ± 3.13

3.3.3 Immunomodulatory properties of BSG-derived fractions

Average TNF-alpha concentrations for all test fractions are presented in Table 3.5. Concentrations ranged between 7.616 ± 2.33 to 9.410 ± 3.45 pg/ml. Reduction of TNF-alpha would be of interest when it acts as a pro-inflammatory agent and based on Figure 3.3 and Table 3.5 it is evident that all BSG-derived fractions did decrease TNF-alpha secretion. However, while the levels of TNF-alpha decreased there was no significant difference between the positive control (Media & Cells & Con A) or BSG intact (CIT-1) and the BSG-derived test fractions. High levels of imprecision must be acknowledged as represented by standard deviation in Figure 3.3, which makes it difficult to interpret the data. It is apparent that BSG-derived fraction CIT-2 overall showed the greatest ability to modulate TNF-alpha levels.

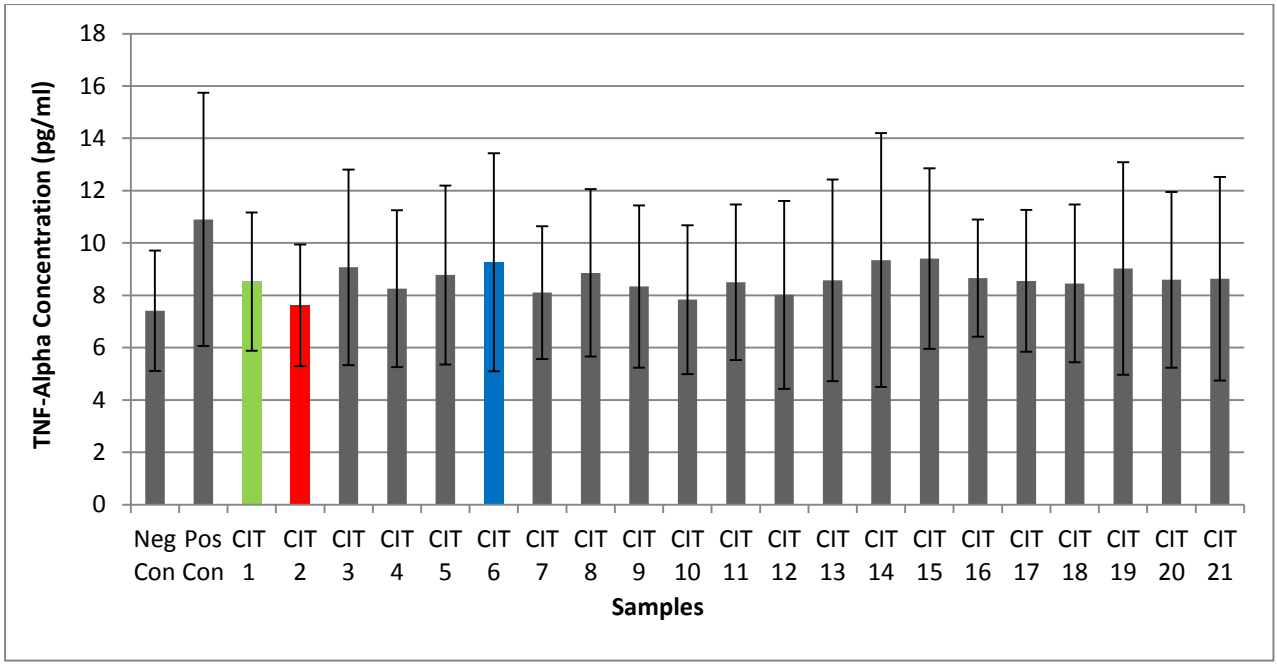

Figure 3.3: Immunomodulatory activity of BSG-derived fractions (CIT-1-CIT-21) following *in vitro* digestion. No significant differences were observed between BSG-derived fractions and CIT-2 (protein) or between BSG-derived fractions and CIT-6 (phenolic). Data represents mean ± standard deviation of TNF-alpha levels measured by ELISA. Results are based on three independent experimental replicates.

Table 3.5: TNF-Alpha Assay Results for BSG-derived fractions (CIT-1–CIT-21) following *in vitro* digestion and comparison of all fractions relative to each other/Multiple Comparisons. Data represents mean ± standard deviation (SD) values based on three independent experiments.

CIT Code	Sample	Average TNF-Alpha Concentration (pg/ml ± SD)
-	Media & Cells	7.412 ± 2.30
-	Media & Cells & Con A	10.903 ± 4.84
CIT-1	BSG Intact	8.525 ± 2.64
CIT-2	Protein Hydrolysate	7.616 ± 2.33
CIT-3	New Phenolic extraction	9.074 ± 3.74
CIT-4	Soluble Fraction	8.251 ± 3.00
CIT-5	Insoluble Fraction	8.778 ± 3.42
CIT-6	Phenolic Rich Fraction	9.266 ± 4.17
CIT-7	Permeate 3kDa	8.105 ± 2.54
CIT-8	Permeate 10kDa	8.860 ± 3.20
CIT-9	Permeate 50kDa	8.337 ± 3.10
CIT-10	Not Separated Fraction	7.834 ± 2.84
CIT-11	Retentate 3kDa	8.502 ± 2.97
CIT-12	Retentate 10kDa	8.017 ± 3.59
CIT-13	Retentate 50kDa	8.573 ± 3.85
CIT-14	BSG Peptide Rich Fraction	9.346 ± 4.85
CIT-15	Old Fibre Rich Fraction	9.410 ± 3.45
CIT-16	New Fibre Rich Fraction	8.659 ± 2.23
CIT-17	Defatted Hydrolysate (d1)	8.550 ± 2.71
CIT-18	Defatted Hydrolysate (d2)	8.456 ± 3.02
CIT-19	Hydrolysate da	9.026 ± 4.07
CIT-20	H20 Extraction	8.593 ± 3.36
CIT-21	BSG Aqueous Extraction	8.633 ± 3.89

3.4 Discussion

BSG is known to contain a significant number of bioactive compounds that have antioxidant and immunomodulatory properties. Incorporating BSG-derived ingredients in functional foods could help design healthy food options (Fărcaş et al., 2014b). For this, it is of interest to identify if modifying the hydrolysis and extraction procedures used in BSG processing could generate more potent bioactive extracts. Identifying extracts with multi-bioactive properties would be of interest in functional food design (Fiore et al., 2019).

The current study investigated if modifying extraction methods could generate novel BSG-derived fractions with improved antioxidant and immunomodulatory properties. These bioactivities were measured following digestion and using *in vitro* cell-based models, as confirming bioactivity of digested fractions in cell-based models is more physiologically relevant and gives more credence to the health-promoting potential of the BSG-derived fractions. Two fractions, a peptide-rich fraction (CIT-14) and interestingly a fraction generated using a simple water extraction method (CIT-20), had significantly higher ($p<0.05$) cellular antioxidant activity compared to the intact BSG-derived fraction (CIT-1). For CIT-14, this finding corroborates earlier data from chemical-based assays (chapter 2), whereby this fraction proved to be one of the most antioxidant BSG-derived fractions (Table 2.4 and Table 2.5). It is evident in Table 3.3 and Table 3.4, following a multiple comparison test, that a minority of BSG-derived fractions presented significantly different ($p<0.05$) cellular antioxidant activities to each other. Interestingly, CIT-14 and CIT-20 were the only two BSG-derived fractions that was significantly different ($p<0.05$) to more than three BSG-derived fractions. Both CIT-4 and CIT-15 proved to be significantly different to CIT-13, CIT-14 and CIT-20 while BSG-derived fractions CIT-1, CIT-5, CIT-7, CIT-9 and CIT-12 presented significantly different cellular antioxidant activity compared to the CIT-14 and CIT-20 (Table

3.3 and Table 3.4). CIT-2, CIT-3, CIT-6, CIT-8, CIT-10, CIT-11, CIT-16, CIT-17, CIT-19 and CIT-21 presented cellular antioxidant activities that proved to have no significant differences to other BSG-derived fractions (Table 3.3 and Table 3.4).

The immunomodulatory properties of BSG have been extensively studied *in vitro* (McCarthy et al., 2013a; McCarthy et al., 2013c). Crowley et al. (2015) examined the effect of incorporating a digested BSG protein hydrolysate into low-fat milk on TNF-α cytokine production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Cian et al. (2020) compared the impact of BSG hydrolysates and digested BSG hydrolysate on TNF-α production in LPS-stimulated spleen macrophages.

TNFα is a pleitropic cytokine as it possesses both pro-inflammatory and anti-inflammatory properties and supports both the adaptive and innate immune response, promotes cell proliferation in conjunction with mediating expression of transcription factors, genes for growth factors, receptors and cytokines (Holbrook et al., 2019; Ye et al., 2018; Ravussin & Smith, 2016; Clark & Vissel, 2015; Pascoe, 2009; Popa et al., 2007; Ksontini et al., 1998). In the current study the data suggested that all of the fractions were immunomodulatory as they all reduced TNFα secretion compared to the positive control, but not significantly. The protein hydrolysate (CIT-2) had the greatest effect and performed better than the other protein-derived fractions, including the defatted and permeate fractions, therefore suggesting that perhaps a combination of bioactive peptide may be contributing to the immunomodulatory effects. It is evident in Table 3.5, following a multiple comparison test, that no BSG-derived fraction presented a significantly different ($p<0.05$) immunomodulatory activity to another BSG-derived fraction, sample control, negative control or positive control.

Further work is needed to clarify if the fractions are associated with pro- or anti-inflammatory effects by measuring other relevant cytokines, such as the pro-inflammatory cytokine INF- γ

(McCarthy et al., 2013a; McCarthy et al., 2013b; McCarthy et al., 2013c) or the anti-inflammatory cytokine IL-6 (Crowley et al., 2015; Scheller et al., 2011).

Removing the fat content of fractions CIT-17 and CIT-18 did not improve their cellular antioxidant and immunomodulatory properties.

A study conducted by Zujko et al. (2015) revealed that older adults, particularly women, have significantly low levels of antioxidants in their diet compared to younger adults. Kopf et al. (2018) reported that consuming whole grain foods can reduce the levels of inflammation biomarkers, such as lipopolysaccharide binding protein (LBP) and TNF-α, and reduce the risk of metabolic disease. The consumption of functional foods containing BSG-derived antioxidant and anti-inflammatory compounds could benefit older adults by lowering inflammation and related conditions, and reduce the risk of coronary heart disease (Kopf et al., 2018; Lefevre & Jonnalagadda, 2012).

3.5 Conclusion

The findings of this study suggest that the cellular antioxidant potential of the BSG-derived fractions was varied and only two fractions demonstrated significantly higher antioxidant potential compared to the intact BSG. A peptide-rich, BSG-derived fraction (CIT-14) displayed the greatest cellular antioxidant potential out of all fractions tested. This fraction was also associated with immunomodulatory properties, but further studies are required to confirm whether the fraction can promote anti- or pro-inflammatory effects. While all fractions reduced TNF-alpha secretion compared to the positive control, eight fractions (CIT-2, CIT-4, CIT-7, CIT-9, CIT-10, CIT-11, CIT-12, CIT-18) reduced TNF-alpha secretion compared to the intact BSG fraction (but not significantly). Further investigations using relevant cytokines such as IL-6 and IFN-γ would be of interest to confirm the anti- and pro-inflammatory properties of these BSG-derived fractions. In addition, it would be of interest to combine some of the more potent fractions to investigate if certain combinations could improve the antioxidant and/or immunomodulatory properties.

Chapter 4

Investigating the Potential *Anti-obesogenic* Properties of BSG-Derived Fractions

4.0 Abstract

Obesity is now considered a global epidemic and is prevalent in older adults. Food-derived bioactive compounds can be associated with anti-obesity properties. In this study the potential anti-obesogenic properties of BSG and BSG-derived fractions was investigated *in vitro* using cell-based and enzyme-based assays. The ability of test fractions to prevent adipocyte differentiation of 3T3-L1 cells and the anti-lipase activity of the fractions were examined. One novel BSG-derived fraction (CIT-20) proved to have the strongest anti-adipogenic activity. The results of this study suggest that BSG-derived fractions are associated with anti-adipogenic properties and thus, this increases the potential of incorporating such fractions as an ingredient in obesity prevention based functional foods that are aimed at the elderly population.

4.1 Introduction

Obesity is the fifth leading cause of death globally (Buchholz & Melzig, 2015), affecting 604 million adults (James, 2018). A body mass index (BMI) of >30 kg/m^2 is classified as obese (Konstantinidi & Koutelidakis, 2019; Mobbs, 2014), with over 300 million people worldwide surpassing this threshold (Dixon, 2010). The World Health Organisation has reported that if trends continue, Ireland will be one of the most overweight countries within Europe by the year 2030, with an astonishing prediction of 85% of women and 89% of men either obese or overweight (O'Shea & Hayes, 2015). Obesity is prevalent in the older adult population (aged ≥ 65 years) (Malenfant & Batsis, 2019; Leahy et al. 2014 ; Mathus-Vliegen et al., 2012; Houston et al., 2009; Villareal et al., 2005). Indeed, reports suggest that weight gain in women peaks around 60 years of age and peaks in men at 50–55 years of age (James, 2018). The incidence of obesity in the older adult population is increasing and in 2018 a National Health and Nutrition Examination Survey in the United States revealed that obesity rates in the 60+ year population was greater than 39.4% in females and 37.5% in males (Batsis & Zagaria, 2018).

Obesity is described as a pathological condition whereby the excessive accumulation of body fat can be associated with a variety of pathological consequences and adverse outcomes (Abdelaal et al., 2017). These can include inflammation, osteoarthritis, type 2 diabetes, sleep apnea, quality of life, cardiovascular disease, cancer, musculoskeletal disorders, dyslipidemia, osteoarthritis, psychological issues, metabolic syndrome and insulin resistance (Procaccini et al., 2019; Jura & Kozak, 2016; Hakkak & Bell, 2016; Kearns et al., 2014; Han et al., 2011; Dixon, 2010; Pi-Sunyer, 2009; Gutierrez et al., 2009). Obesity is a multifactorial

disease that also escalates the risk of premature mortality (Karri et al., 2019; Smethers & Rolls, 2018; Amarya et al., 2014).

Many factors influence global obesity including food environment, urbanization, cultural globalization, physical activity reduction and economic globalization (Fox et al., 2019; Romieu et al., 2017) yet diet plays an essential role in maintaining a healthy body weight and reducing obesity (Konstantinidi & Koutelidakis, 2019; Fock & Khoo, 2013; Makris & Foster, 2011). A healthy diet is key to weight management and should be harmonized with regular physical exercise (Karri et al., 2019; Mohamed et al., 2014; Fock & Khoo, 2013). It is important to consider the amount of food consumed as well as the type of foods being consumed (Rolls, 2017). Several studies have reported on the anti-obesity properties of plant-derived foods (Ojulari et al., 2020; Liu et al., 2020; Kim et al., 2020; Tung et al., 2016; Lee et al., 2016; Wang et al., 2014; Mohamed et al., 2014) and whole grains have been linked to body weight regulation and obesity reduction (Maki et al., 2019; Karl & Saltzman, 2012; Park et al., 2011). These foods are a natural source of bioactive compounds, such as phytochemicals like tricin which have proven to suppress adipogenesis and lipid accumulation (Lee et al., 2015). Whole grains are associated with modifying critical measures linked to obesity, specifically waist circumference, body mass index and weight, and promote satiety effects (Develaraja et al., 2016; Mikušová et al., 2010).

At a cellular level, obesity is defined as an escalation in number and size of mature adipocytes within adipose tissue (Haider & Larose, 2019; Valii et al., 2018; Parlee et al., 2014). Datta et al. (2017) and Ruiz-Ojeda et al. (2016) described adipogenesis as the formation of preadipocytes into mature adipocytes by means of differentiation. Adipogenesis is influenced by several exogenous and/or endogenous factors (Stoecker et al., 2017). One potential strategy to combat overweight and obesity is to modulate lipid metabolism by limiting adipocyte differentiation and/or promoting adipolysis or inhibiting lipase activity

(Haider & Larose, 2019; Kim et al., 2016a; Alam et al., 2016). Lim et al. (2014) reported that germinated brown rice extracts demonstrated pancreatic lipase inhibitory properties, stimulated adipocyte lipolysis and inhibited adipogenesis in 3T3-L1 cells, thus signifying the strong anti-obesity activity of the germinated brown rice extracts.

Research completed by Lee et al. (2015) reported that extracts of oat hull exhibited anti-adipogenic properties. The oat hull extract was shown to decelerate lipid synthesis and lipogenesis, inhibiting adipocyte fatty acid accumulation and modulating messenger ribonucleic acid (mRNA) expression of specific transcriptional factors [CEBP/α, peroxisome proliferator-activated receptor gamma (PPAR-γ) and sterol regulatory element-binding protein 1 (SREBP1)] linked to adipocyte metabolism (Lee et al., 2015). Park et al. (2011) investigated the effect of nine Korean cereal extracts on the differentiation of a mouse fibroblast preadipocyte line (3T3-L1 cells) and their impact on the expression of relevant transcription factors. The oil red O method used, revealed that several extracts demonstrated anti-adipogenic activity and were found to downregulate the adipogenic transcription factors SREBP1, C/EBPα and PPARγ (Park et al., 2011; Sul et al., 1998).

A more recent study by Kim et al. (2019) revealed that rice hull extract (RHE) suppressed the expression of adipogenesis linked genes in 3T3-L1 cells and high-fat diet-induced obese mice. RHE also had a positive impact on LDL-cholesterol and triglyceride (TG) serum levels and the 4-hydoxybenzaldehyde compound of the RHE was shown to impact adipogenesis in both adipose and liver tissue (Kim et al., 2019).

Balanced lipase metabolism is vital to preserve homeostasis, however an imbalance will result in hyperlipidemia or obesity (Birari & Bhutani, 2007). The chief lipolytic enzyme is pancreatic lipase, which hydrolyses 50-70% of total dietary fats and requires colipase to fully function (Birari & Bhutani, 2007). Pancreatic lipase inhibition aids in the prevention of

dietary triglyceride absorption and fat absorption, thus reducing the likelihood of obesity (Birari & Bhutani, 2007). Orlistat, a lipase inhibitor, is the most widely employed pharmacologic treatment for obesity in Europe and proven to inhibit pancreatic lipase activity *in vivo*, however it has been shown to have negative gastrointestinal side effects (Buchholz & Melzig, 2015; Birari & Bhutani, 2007). Natural effective pancreatic lipase inhibitors have also been identified (Jeepipalli et al., 2020) and include food-derived phenolic compounds such as hydroxycinnamic acids, lignans, flavonoids and hydroxybenzoic acids (Fabroni et al., 2016; Buchholz & Melzig, 2015; Mohamed et al., 2014; Lunagariya et al., 2014; Birari & Bhutani, 2007). These polyphenols can inhibit pancreatic lipase and lipoprotein lipase activity and have demonstrated the ability to reduce plasma free fatty acid concentrations, fat weight as well as body weight (Buchholz & Melzig, 2015; Mohamed et al., 2014).

It would be of interest to identify additional natural foods or food ingredients that can inhibit lipase activity and incorporate them in the design of functional foods that could help combat obesity. BSG is known to contain a significant number of bioactive compounds, including a range of phenolic compounds, but there are yet no studies to date that have investigated the potential anti-obesogenic properties of BSG. This study aims to evaluate the *in vitro* anti-obesogenic properties of novel BSG-derived fractions using the 3T3-L1 preadipocyte cell culture model and lipase inhibition assays.

4.2 Materials and Methods

4.2.1 Materials

Reagents sourced from Sigma-Aldrich: Dulbecco's Modified Eagle Medium (DMEM), Penicillin-Streptomycin, L-Glutamine Solution, Phosphate buffered saline, Thiazolyl Blue Tetrazolium Bromide, Dimethyl Sulfoxide (DMSO), 3-Isobutyl-1-methylxanthine (IBMX), Dexamethasone (DEX), Insulin Solution Human (Insulin), Rosiglitazone, Lipase from Porcine Pancreas (Lipase), Orlistat, 4-Nitrophenyl butyrate (NPB), Tween 80 Buffer, Ethanol, Potassium phosphate monobasic, Adipogenesis Assay kit. **Reagents sourced from ThermoFisher Scientific:** Gibco New Born Calf Serum (NBCS).

4.2.2 BSG Fractions

BSG (Diageo) was processed and fractionated according to the methods described in Section 2.2.2, generating 21 BSG-derived fractions CIT-1-CIT-21 (Table 2.1). These fractions were then digested as outlined in section 2.2.3 and these digestates were further investigated to examine their potential anti-obesogenic properties.

4.2.3 Cell Culture

The mouse embryo preadipocyte cell line 3T3-L1 (ATCC® CRL-1658™) was cultured and maintained, in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% v/v new born calf serum (NBCS), 1% v/v Penicillin-Streptomycin (Pen-Strep) and 1% v/v L-glutamine. For experiments 3T3 cells were used between passage number 5 and 20.

The 3T3-L1 cell model is the most characterised and well-established *in vitro* cell model to assess the impact of nutrients on adipogenesis and obesity-related properties (Ruiz-Ojeda et al., 2016; Kawai & Rosen, 2013). More than 5000 published articles concerning 3T3-L1 adipocyte biochemistry and adipogenesis have been generated (Zebisch et al., 2012). Caution

was taken to ensure that young cultured 3T3-L1 cells were utilized for adipogenesis experiments as differentiation is significantly impacted by increased passage number and over confluent cells (Ruiz-Ojeda et al., 2016; Zebisch et al., 2012).

4.2.4 Cell Cytotoxicity Investigations

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to identify sub-toxic concentrations of the BSG fractions (Ho et al., 2012). Cells were adjusted to a density of $2x10^5$cells per ml in antibiotic-free medium (DMEM) supplemented with 10% new born calf serum (NBCS) and 50µl of the cell suspension was seeded into the appropriate number of test wells in a 96-well plate. Cells were then incubated for 24hrs at 37°C, 5% CO_2. Following incubation, the media was removed and replaced with 50µl of fresh antibiotic-free media (DMEM) and test BSG digestate fractions were added at concentrations of 0, 0.5, 1, 2, 2.5, 5 & 10% (v/v), such that the final volume in each well was 100µl. The plate was re-incubated for a further 48hrs at 37°C, 5% CO_2.

Following this, all media was removed and 100µl of fresh DMEM media (minus the NBCS) and 10µL of MTT reagent (5mg/1ml PBS) was added to each well and the plate was then wrapped with foil and incubated for 4hr at 37°C. The contents were then removed and 100 µL of DMSO was added to each well. Absorbance was measured spectrophotometrically using a microplate reader (Varioskan™ LUX multimode microplate reader) at 595nm. A media blank (no cells) and control blank (DMSO only) was used to account for background colour of the BSG digestate fractions.

4.2.5 Adipogenesis Assay - Measurement of Triglyceride accumulation

The potential of the BSG-derived fractions to alter lipid accumulation in cultured 3T3-L1 adipocytes was investigated using a commercial adipogenesis assay kit (Sigma-Aldrich Ltd)

and a method previously described by Ho et al. (2012) with modifications. 2mls of 3T3-L1 cells at $1x10^5$ cells/ml in DMEM (10% FBS) were added to each well of a 6 well plate and incubated for 48hrs. After 2 days, the media was removed and adipogenesis was initiated by the addition of four pro-differentiation agents into the growth media, including 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX), rosiglitazone and insulin (Ruiz-Ojeda et al., 2016; Morrison & McGee, 2015; Zebisch et al., 2012). A 5X stock of the differentiation media (10ml) was initially prepared (Table 4.1A) and sterile filtered.

Table 4.1A: Main components of Adipogenesis Assay

Media component	Stock concentration	Final concentration (cells)	5X Differentiation Media (10mls)
IBMX	≥99% (HPLC) Powder	0.5mM	**0.056g**
DEX	1mg/mL	0.25mM	**5µL**
Insulin	10mg/mL	1µg/ml	**5µL**
Rosiglitazone	1mg/mL	2µM	**36µL**
Media	5x	1x	**9.954mL**

The 5X stock was diluted 1 in 5 with medium (DMEM, 10% FBS) to generate a 1X working solution. Each BSG-derived fraction was mixed with the 1X working differentiation media solution (1% v/v) to a final volume of 2mls and added to the fully confluent 3T3-L1 preadipocytes within appropriately labelled 6-well plates. Sample exposure only occurred once. The positive control consisted of cells only and 2mls of the working differentiation media solution (1X). After 2 days of incubation, the differentiation media and samples were removed and 2mls of fresh DMEM, 10% FBS with 10µg/mL insulin (1µL stock insulin/mL DMEM with FBS) was added to each well. This media was changed and replaced with 2mls of fresh DMEM, 10% FBS and 10µg/mL insulin, without the samples every 2 days. Lipid accumulation was monitored microscopically and visible roughly four days after exposure to the differentiation media (Figure 4.1) as triglyceride synthesis and glucose uptake is

intensified (Morrison & McGee, 2015; Shao et al., 2013). On day 10, all media was aspirated from the 6 well plates and cells were washed twice with cold PBS. The cells were scraped in 1ml of fresh cold PBS and relocated to sterile eppendorf tubes, prior to centrifugation for 5mins at 15,000rpm. The supernatant was removed, and the pellet was resuspended in 200µL of lipid extraction buffer for triglyceride analysis. Total cellular concentrations of triglycerides was determined using the Adipogenesis Assay Kit - MAK040 (Sigma-Aldrich, 2019). Each test sample was analysed in duplicate and the experiment repeated twice.

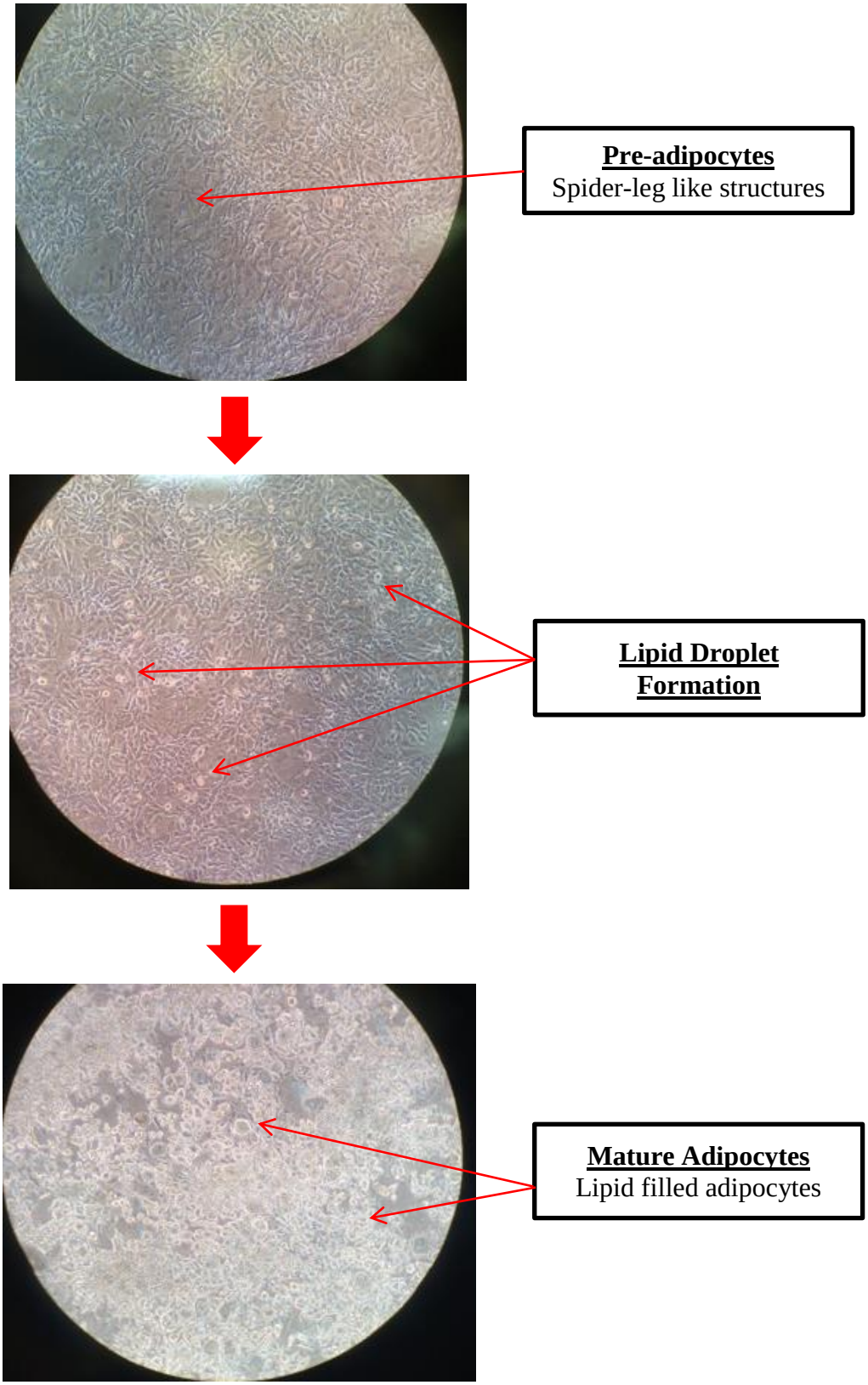

Figure 4.1: Microscopic image showing different cell morphologies captured at the different steps of the differentiation process - pre-adipocytes, differentiating 3T3-L1 cells and fully differentiated/mature 3T3-L1 adipocytes in a 6 well plate

4.2.6 Lipase Inhibition Assay

Natural lipase inhibitors have therapeutic potential in the management of obesity (Liu et al., 2020; Karri et al., 2019; Mhatre et al., 2016; Sahib et al., 2012; De la Garza et al., 2011). The digested BSG-derived fractions were investigated to determine if they could inhibit the lipolytic activity of pancreatic lipase.

The lipase inhibition activity of the BSG-derived fractions was assessed as previously described (Roh & Jung, 2012; Zheng et al., 2010; Lee et al., 2010) with modifications. Briefly, lipase stock (1mg/mL) was prepared in 0.1mM potassium phosphate buffer 1 (pH 6.0). 100µl BSG-derived samples (0.5%) were pre-incubated with the 100µl lipase enzyme for one hour at 30ºC in potassium phosphate buffer 2 (0.1mM, pH 7.2, combined with 0.1% Tween 80). Following incubation, 100µL of substrate, 25mM 4-Nitrophenyl butyrate (p-NPB) was added to each test BSG-derived sample and to reach a final volume of 1mL, potassium phosphate buffer 2 (0.1mM, pH 7.2, combined with 0.1% Tween 80) was added to each eppendorf tube before a 5 minute incubation at 30ºC. The amount of p-nitrophenyl butyrate released in the reaction was read immediately after the 5 minute incubation period at 405nm using a UV–Vis spectrophotometer (UV-1800, SHIMADZU EUROPA, Shimadzu Schweiz GmbH). The positive control included Orlistat (50µg/mL), a known lipase inhibitor (Irondi et al., 2019; Seyedan et al., 2015; Roh & Jung, 2012; Heck et al., 2000). The negative control with and without inhibitor was also determined. The percentage lipase inhibitory activity (I%) was calculated according to the following formula:

$$\text{Inhibitory activity (I \%)} = (\text{Ac- As} / \text{Ac}) \times 100$$

Where **Ac** is the absorbance of the control and **As** is the absorbance of the sample (Maqsood et al., 2017).

Table 4.1B: Lipase Inhibition Assay - BSG Sample Set up

Sample/Orlistat	100µl
Lipase	100µl
Tween Buffer (Buffer 2)	700µl
Substrate	100µl
Total Volume	**1000µl (1ml)**

Table 4.1C: Lipase Inhibition Assay - Control Set up

B= Buffer, **E** = Enzyme, **I** = Inhibitor, **S** = Substrate

Control	Components	Description
A	**E**(100ul) & **B** (800ul) & **S** (100ul)	Activity of the enzyme without inhibitor
a	**B** (900ul) & **S** (100ul)	Negative control without inhibitor
B	**I** (100ul) & **E** (100ul) & **B** (700ul) & **S** (100ul)	Activity of the enzyme with inhibitor
b	**I** (100ul) & **B** (800ul)& **S** (100ul)	Negative control with inhibitor

4.2.7 Statistical Analysis

The bioactivities of each BSG sample/fraction (following *in vitro* digestion) were analysed in three independent experiments. Data presented represents the average ± standard deviation of all measurements. Statistical analysis was carried out using the IBM Statistical Package for Social Sciences (SPSS v.26). A one-way analysis of variance (ANOVA) was used to compare differences in the bioactivity between all BSG digestates. Controlling for multiple comparisons, the Dunnett's post-hoc test was used to evaluate mean changes between digestates and a control sample. A p-value <0.05 was considered statistically significant (Appendices, Pages 216-217; 218-219)

4.3 Results

4.3.1 Cell Cytotoxicity

The digested BSG-derived fractions were screened against cultured preadipocyte cells for their cytotoxic effects to identify concentrations that could be used in the adipogenesis assay. Samples CIT-1 – CIT-21 reduced 3T3 cell proliferation, however not all in a dose-dependent manner, and certain samples stimulated growth (Table 4.2). For further experiments, a non-toxic concentration of 1% (v/v) sample was selected as the majority (18/21; >85%) samples were associated with viability of ≥80% with the remaining three samples having viability ≥70%.

Table 4.2: Effect of novel BSG-derived fractions (0-5%) on cell proliferation (% of control) in the 3T3 cell line following *in vitro* digestion

Sample	CIT Code	0%	0.5%	1%	2%
BSG Intact	CIT-1	100.00	76.65	77.04	60.94
Protein Hydrolysate	CIT-2	100.00	119.41	110.25	85.62
New Phenolic Extraction	CIT-3	100.00	87.86	95.52	80.27
Soluble Fraction	CIT-4	100.00	79.31	90.52	59.19
Insoluble Fraction	CIT-5	100.00	106.05	118.15	73.85
Phenolic Rich Fraction	CIT-6	100.00	72.54	67.20	18.87
Permeate 3kDa	CIT-7	100.00	89.93	86.53	84.64
Permeate 10kDa	CIT-8	100.00	92.39	78.28	74.03
Permeate 50kDa	CIT-9	100.00	91.87	83.63	64.06
Not Separated Fraction	CIT-10	100.00	110.85	126.30	98.91
Retentate 3kDa	CIT-11	100.00	80.89	80.41	75.22
Retentate 10kDa	CIT-12	100.00	92.82	98.55	87.97
Retentate 50kDa	CIT-13	100.00	66.09	87.20	49.07
BSG Peptide Rich Fraction	CIT-14	100.00	100.51	89.62	69.62
Old Fibre Rich Fraction	CIT-15	100.00	89.88	89.15	94.82
New Fibre Rich Fraction	CIT-16	100.00	85.85	86.75	72.33
Defatted Hydrolysate (d1)	CIT-17	100.00	113.13	92.60	55.24
Defatted Hydrolysate (d2)	CIT-18	100.00	107.41	107.15	77.73
Hydrolysate da	CIT-19	100.00	72.65	69.93	58.55
H20 Extraction	CIT-20	100.00	84.54	82.13	75.32
BSG Aqueous Extraction	CIT-21	100.00	94.18	87.90	81.74

4.3.2 Measurement of lipid accumulation based on triglyceride content

There was a distinct difference in the appearance of the confluent preadipocyte cells and the mature adipocytes/fully differentiated 3T3-L1 cells, with lipid droplets visible in the mature adipocytes (Figure 4.1). Microscope examination suggested that the number and size of lipid droplets was altered in 3T3-L1 adipocytes treated with the test BSG-derived fractions, which was confirmed by results of the triglyceride assay.

The lowest triglyceride concentration was recorded in the negative control (0.165 ± 0.04 nmol/well), while the positive control had the largest triglyceride concentration (2.464 ± 0.24 nmol/well), signifying the formation of lipid droplets and triglyceride accumulation during the differentiation process. Initially, the ability of CIT-1 (BSG intact), CIT-2 (protein hydrolysate) and CIT-6 (phenolic fraction) to modulate adipocyte differentiation was investigated and all three fractions significantly reduced ($p<0.05$) lipid accumulation during the differentiation process compared to the positive control (Figure 4.1A). Although CIT-2 demonstrated the stronger anti-adipogenic effect, there was no significant difference in triglyceride concentration between CIT-1, CIT-2 and CIT-6 (Figure 4.2). The effects of all BSG-derived digestate fractions on fat droplet formation in 3T3-L1 cells are presented in Figure 4.2B and Table 4.3.

Triglyceride concentrations ranged from 0.679 ± 0.21 to 1.646 ± 0.25 nmol/well with all fractions causing a significant reduction ($p<0.05$) in lipid accumulation compared to the positive control, demonstrating that all fractions impacted the differentiation process. Fractions CIT-8, CIT-13 and CIT-15 were associated with the weakest anti-adipogenic activity, decreasing adipocyte differentiation by 33.2%, 41.9% and 43.8%, respectively, while fraction CIT-20 exhibited the greatest anti-adipogenic activity followed by CIT-17 and CIT-4, decreasing adipocyte differentiation by 72.4%, 71.3% and 68.3%, respectively. CIT-4,

CIT-17 and CIT-20 showed significantly lower ($p<0.05$) triglyceride accumulation to CIT-1 while BSG-derived fractions CIT-8 and CIT-13 revealed significantly higher ($p<0.05$) triglyceride contents compared to CIT-1 (Table 4.3 and Figure 4.2B).

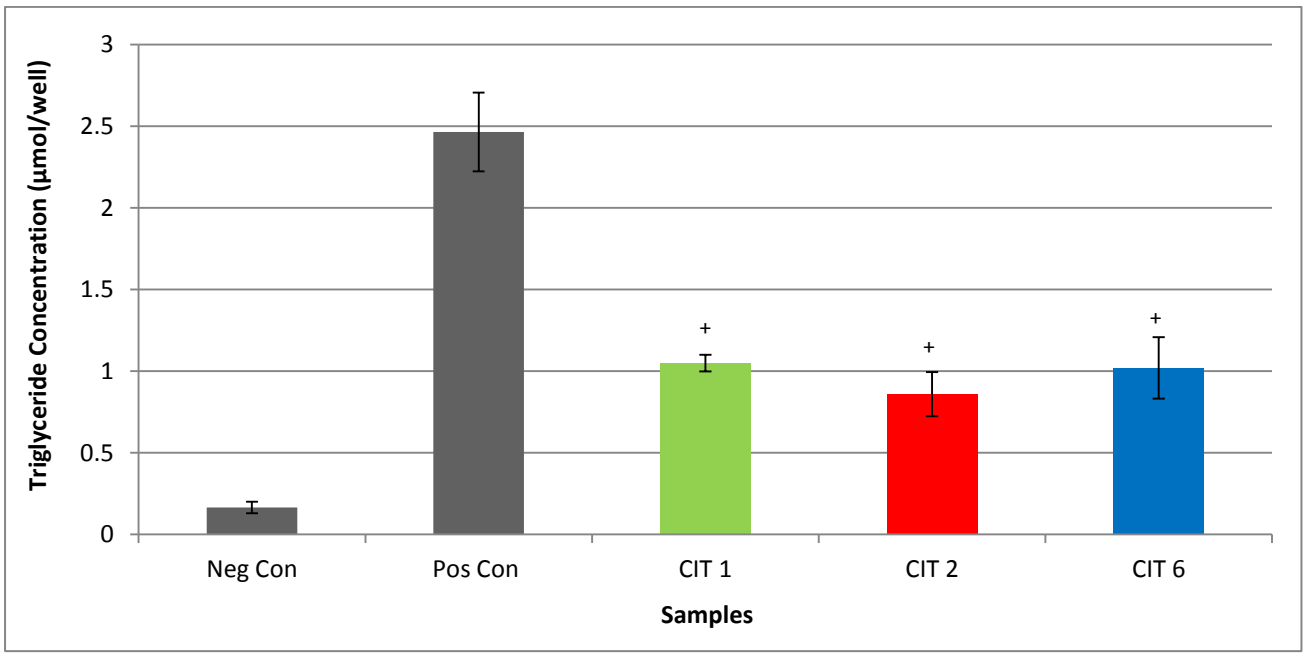

Figure 4.2A: Triglyceride Concentrations of BSG-derived fractions, CIT-1 vs. CIT-2 and CIT-6 following *in vitro* digestion. $^{+}$ denotes a statistically significant ($p<0.05$) difference between fully differentiated 3T3 cells (positive control/Pos Con) and BSG-derived fractions. No significant differences were observed between Sample Control (CIT-1) and CIT-2 (protein) or between Sample Control (CIT-1) and CIT-6 (phenolic). Data represents mean ± standard deviation from two independent experimental replicates.

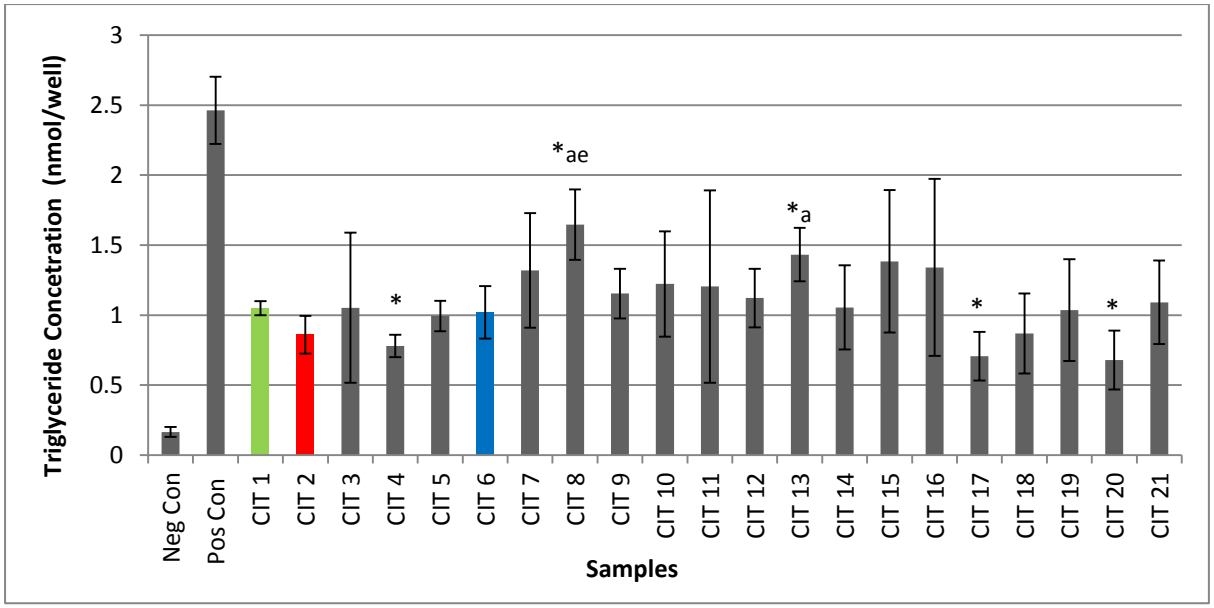

Figure 4.2B: Triglyceride Concentrations of BSG-derived fractions (CIT-1-CIT-21) following *in vitro* digestion. $^{+}$ denotes a statistically significant ($p<0.05$) difference between fully differentiated 3T3 cells (positive control/Pos Con) and BSG-derived fractions. * Denotes a significant difference ($p<0.05$) in CIT-1 (BSG intact fraction)/Sample Control and BSG-derived digested fractions. ^{a} denotes a statistically significant ($p<0.05$) difference between BSG protein fraction (CIT-2) and BSG-derived fractions. ^{e} denotes a statistically significant ($p<0.05$) difference between BSG phenolic fraction (CIT-6) and BSG-derived fractions. Data represents mean ± standard deviation from two independent experimental replicates.

Table 4.3: Adipogenesis Assay – Effect of BSG-derived fractions following *in vitro* digestion on the differentiation of 3T3-L1 cells and measurement of lipid accumulation based on triglyceride content. Data represents mean ± standard deviation (SD) values based on three independent experiments. * Denotes a significant difference ($p<0.05$) between the BSG-derived digested fractions and intact BSG (CIT-1)/Sample Control. [a] denotes a statistically significant ($p<0.05$) difference between BSG protein fraction (CIT-2) and BSG-derived fractions. [e] denotes a statistically significant ($p<0.05$) difference between BSG phenolic fraction (CIT-6) and BSG-derived fractions.

Sample	**CIT Code**	**Adipogenesis Assay *Average Triglyceride Concentration* (nmol/well ± SD)**	**% Decrease in Adipocyte Differentiation *Triglyceride Concentration***
Negative Control	-	0.165 ± 0.04	-
Positive Control	-	2.464 ± 0.24	-
BSG Intact	CIT-1	1.049 ± 0.05	57.4
Protein Hydrolysate	CIT-2	0.859 ± 0.13	65.1
New Phenolic Extraction	CIT-3	1.052 ± 0.54	57.3
Soluble Fraction	CIT-4	0.780 ± 0.08*	68.3
Insoluble Fraction	CIT-5	0.993 ± 0.11	59.7
Phenolic Rich Fraction	CIT-6	1.019 ± 0.19	58.6
Permeate 3kDa	CIT-7	1.319 ± 0.41	46.5
Permeate 10kDa	CIT-8	1.646 ± 0.25*[ae]	33.2
Permeate 50kDa	CIT-9	1.154 ± 0.18	53.2
Not Separated Fraction	CIT-10	1.222 ± 0.38	50.4
Retentate 3kDa	CIT-11	1.204 ± 0.69	51.1
Retentate 10kDa	CIT-12	1.121 ± 0.21	54.5
Retentate 50kDa	CIT-13	1.432 ± 0.19*[a]	41.9
BSG Peptide Rich Fraction	CIT-14	1.055 ± 0.30	57.2
Old Fibre Rich Fraction	CIT-15	1.384 ± 0.51	43.8
New Fibre Rich Fraction	CIT-16	1.341 ± 0.63	45.6
Defatted Hydrolysate (d1)	CIT-17	0.706 ± 0.17*	71.3
Defatted Hydrolysate (d2)	CIT-18	0.869 ± 0.29	64.7
Hydrolysate da	CIT-19	1.035 ± 0.36	58.0
H20 Extraction	CIT-20	0.679 ± 0.21*	72.4
BSG Aqueous Extraction	CIT-21	1.091 ± 0.30	55.7

Table 4.4: Adipogenesis Assay – Measurement of lipid accumulation based on triglyceride content and comparison of all fractions relative to each other (CIT-2-CIT-21)/Multiple Comparisons following *in vitro* digestion. Data represents mean ± standard deviation (SD) values based on three independent experiments. Significant difference ($p<0.05$) in antioxidant activity are denoted by small letter superscripts (a-t) and are represented with the following associations: **Positive Control**=+; **CIT-2**=a; **CIT-3**=b; **CIT-4**=c; **CIT-5**=d; **CIT-6**=e; **CIT-7**=f; **CIT-8**=g; **CIT-9**=h; **CIT-10**=i; **CIT-11**=j; **CIT-12**=k; **CIT-13**=l; **CIT-14**=m; **CIT-15**=n; **CIT-16**=o; **CIT-17**=p; **CIT-18**=q; **CIT-19**=r; **CIT-20**=s; **CIT-21**=t

Sample	CIT Code	Adipogenesis Assay *Average Triglyceride Concentration* (nmol/well ± SD)
Positive Control	-	2.464 ± 0.24
Protein Hydrolysate	CIT-2	$0.859 \pm 0.13^{+gl}$
New Phenolic Extraction	CIT-3	$1.052 \pm 0.54^{+}$
Soluble Fraction	CIT-4	$0.780 \pm 0.08^{+ghl}$
Insoluble Fraction	CIT-5	$0.993 \pm 0.11^{+gl}$
Phenolic Rich Fraction	CIT-6	$1.019 \pm 0.19^{+g}$
Permeate 3kDa	CIT-7	$1.319 \pm 0.41^{+}$
Permeate 10kDa	CIT-8	$1.646 \pm 0.25^{+acdekpqs}$
Permeate 50kDa	CIT-9	$1.154 \pm 0.18^{+cps}$
Not Separated Fraction	CIT-10	$1.222 \pm 0.38^{+}$
Retentate 3kDa	CIT-11	$1.204 \pm 0.69^{+}$
Retentate 10kDa	CIT-12	$1.121 \pm 0.21^{+g}$
Retentate 50kDa	CIT-13	$1.432 \pm 0.19^{+acdpqs}$
BSG Peptide Rich Fraction	CIT-14	$1.055 \pm 0.30^{+}$
Old Fibre Rich Fraction	CIT-15	$1.384 \pm 0.51^{+}$
New Fibre Rich Fraction	CIT-16	$1.341 \pm 0.63^{+}$
Defatted Hydrolysate (d1)	CIT-17	$0.706 \pm 0.17^{+ghl}$
Defatted Hydrolysate (d2)	CIT-18	$0.869 \pm 0.29^{+gl}$
Hydrolysate da	CIT-19	$1.035 \pm 0.36^{+}$
H20 Extraction	CIT-20	$0.679 \pm 0.21^{+ghl}$
BSG Aqueous Extraction	CIT-21	$1.091 \pm 0.30^{+}$

4.3.3 Lipase Inhibitory effects of BSG-derived digestate fractions

The % lipase inhibitory activities of BSG-derived fractions were compared to the intact BSG (CIT-1), and the BSG-derived fractions CIT-2 and CIT-6. The data is presented in Table 4.5 and signifies that none of the BSG-derived fractions demonstrated lipase inhibition activity using the current method of investigation. However, it is important to acknowledge that the imprecision (as measured by standard deviation) was high for all samples tested (Table 4.5). This data was based on three independent experimental repeats, but the poor precision suggests that an alternative assay should be investigated to examine if more reliable data could be generated.

Table 4.5: Lipase Inhibition (% Inhibition activity) of BSG-derived fractions (CIT-1-CIT-21) following *in vitro* digestion and comparison of all fractions relative to each other/Multiple Comparisons. Data represents mean ± standard deviation (SD) values based on three independent experiments.

CIT Code	Sample	Average % Lipase Inhibition Activity (µg/ml ± SD)
CIT-1	BSG Intact	-24.994 ± 31.32
CIT-2	Protein Hydrolysate	-27.203 ± 34.86
CIT-3	New Phenolic Extraction	-49.373 ± 34.82
CIT-4	Soluble Fraction	-30.529 ± 33.25
CIT-5	Insoluble Fraction	-41.223 ± 42.19
CIT-6	Phenolic Rich Fraction	-25.037 ± 30.74
CIT-7	Permeate 3kDa	-28.471 ± 42.54
CIT-8	Permeate 10kDa	-33.579 ± 36.84
CIT-9	Permeate 50kDa	-35.574 ± 36.66
CIT-10	Not Separated Fraction	-46.387 ± 29.77
CIT-11	Retentate 3kDa	-25.913 ± 31.94
CIT-12	Retentate 10kDa	-27.932 ± 29.03
CIT-13	Retentate 50kDa	-29.477 ± 30.50
CIT-14	BSG Peptide Rich Fraction	-20.805 ± 16.11
CIT-15	Old Fibre Rich Fraction	-15.723 ± 24.82
CIT-16	New Fibre Rich Fraction	-10.267 ± 36.89
CIT-17	Defatted Hydrolysate (d1)	-45.027 ± 39.54
CIT-18	Defatted Hydrolysate (d2)	-41.802 ± 40.49
CIT-19	Hydrolysate da	-45.636 ± 34.75
CIT-20	H20 Extraction	-55.178 ± 34.69
CIT-21	BSG Aqueous Extraction	-61.353 ± 32.33

4.3 Discussion

On a global scale, obesity is increasing and is prevalent in the older adult population (Malenfant & Batsis, 2019; Batsis & Zagaria, 2018; Porter Starr, 2016). Obesity is a complex, multifactorial disorder (Karri et al., 2019) and thus, there isn't just one single solution that would address all contributing factors. However, there are recommended strategies to help combat obesity, and the complications associated with obesity, which usually include a combination of moderate exercise and therapeutic interventions that can impact weight management (Higuera-Hernández et al., 2018; Apovian et al., 2015; Donnelly et al., 2009). Currently, there are several conventional anti-obesity pharmaceutical drugs available, but these are often associated with harmful and unpleasant side effects (Karri et al., 2019). Therefore, there is a need to develop natural, safe and effective strategies and products to help combat overweight and obesity. One strategy is to use natural food sources to develop functional foods that promote satiety (Alviña & Araya, 2016; Rebello et al., 2014; Van Kleef et al., 2012; Halford & Harrold, 2012) and/or impact processes linked to lipid metabolism, such as fat absorption and adipocyte modulation (Yildiz et al., 2020; Ojulari et al., 2020; Chang & Kim, 2019; Valli et al., 2018; Mohamed et al., 2014). Bioactive food compounds have been identified as natural and favourable therapeutic tools to combat obesity, with less harmful side effects (Jayarathne et al., 2017; Sun et al., 2016; Trigueros et al., 2013).

There is significant evidence that foods such as fruit and vegetables can modulate adipocyte metabolism *in vitro* (Mir et al., 2019; Jayarathne et al., 2017; Park et al., 2016; Kang et al., 2016; Williams et al., 2013; Song et al., 2013; Zhang et al., 2012; Jeong et al., 2011). The compounds that are responsible for these effects include resveratrol in grape skin extracts (Aguirre et al., 2014; Zhang et al., 2012; Jeong et al., 2011), polyphenols (hydrocinnamic acids, flavonoids, and proanthocyanidins) in blueberry peel extracts (Song et al., 2013) and

stilbenes, phenolic acids, lignans and flavonoids (like rosmarinic acid, estragole, crocin, menthiol, 6-methoxy luteolin and leosibirin) in selected medicinal herbs (Park et al., 2016). Obesity is correlated with the extent of lipolysis, intracellular lipid accumulation and adipocyte differentiation (Valli et al., 2018), thus foods that impact these processes are of interest.

To the best of our knowledge this is the first study that investigates the ability of BSG and BSG-derived fractions to modulate adipocyte metabolism. Data suggested that BSG and its components decreased triglyceride accumulation *in vitro* during the differentiation process. This effect was not due to reduction in cell viability as the cells were monitored microscopically and the concentrations used were considered non-toxic. All of the fractions significantly reduced ($p<0.05$) triglyceride concentration, due to decreased adipocyte differentiation, and five fractions (CIT-4, CIT-8, CIT-13, CIT-17 and CIT-20) were significantly different to the intact BSG sample.

It is evident in Table 4.3 and Table 4.4, that all 21 BSG-derived fractions modulated adipocyte differentiation, following the lysing (scraping) and centrifugation of cells after a 10 day differentiation period and measuring total cellular concentrations of triglycerides (nmol/well) using the Adipogenesis Assay Kit - MAK040 (Sigma-Aldrich, 2019). The pellet was resuspended in lipid extraction buffer prior to the addition of a lipase enzyme solution and master reaction mix and absorbance was measured at 570nm using a microplate reader.

Following a multiple comparison test, all BSG-derived fractions presented significantly different ($p<0.05$) impacts on cellular triglyceride concentration in comparison to the positive control (fully differentiated 3T3 cells). Five BSG-derived fractions presented significantly different ($p<0.05$) impacts on cellular triglyceride concentration in comparison to the intact BSG (CIT-1), two fractions significantly different ($p<0.05$) to the protein fraction (CIT-2)

and one fraction significantly different ($p<0.05$) to the phenolic fraction (CIT-6) (Table 4.3 and Table 4.4). CIT-8 presented a significantly different ($p<0.05$) impact on cellular triglyceride concentration to the positive control and sample control (CIT-1) as well as eight other BSG-derived fractions (Table 4.3 and Table 4.4). BSG-derived fractions CIT-3, CIT-7, CIT-10, CIT-11, CIT-14, CIT-15, CIT-16, CIT-19 and CIT-21 presented an impact on cellular triglyceride concentrations, yet proved to have no significant difference to the impact of any other BSG-derived fraction (Table 4.3 and Table 4.4).

Adipocyte differentiation is influenced by both environmental and genetic factors and inhibiting this process has potential as a strategy to prevent and treat obesity (Valli et al., 2018). However, further studies are needed to identify how the BSG-derived fractions influence the differentiation process and elucidate the mechanisms involved. Rebollo-Hernanz et al. (2019) demonstrated that phenolic compounds isolated from coffee extracts reduced lipid accumulation *in vitro*, with some of the compounds inhibiting lipid accumulation by 50%. Aranaz et al. (2019) also reported that phenolic compounds can modulate adipocyte differentiation and inhibit the early stages of the process by downregulating the expression of adipogenic genes and transcription factors.

The BSG intact sample and the phenolic fractions in the current study inhibited lipid accumulation by >50%. It would therefore be of interest to further investigate and confirm how these polyphenolic-containing fractions impacted the differentiation process at the molecular level. The impact of food-derived proteins and peptides on adipocyte differentiation can vary depending on the protein/peptide source. Dairy proteins have been reported to promote the differentiation process and lipid accumulation (D'Souza et al, 2020), while certain grain crops like quinoa have been shown to inhibit adipogenesis (Shi et al., 2019). In the current study all of the protein and peptide fractions reduced lipid accumulation

in vitro, with one of the most potent fractions being a defatted hydrolysed extract that decreased adipocyte differentiation by 71%.

None of the BSG test samples inhibited lipase activity and statistical data indicated high levels of impression, thus further studies are required to validate the results. It is evident in Table 4.5, following a multiple comparison test that no BSG-derived fraction presented significantly different ($p<0.05$) lipase inhibition activity to other BSG-derived fractions or sample control. Irondi et al. (2019) reported that both raw and roasted red sorghum grains flours possessed pancreatic lipase inhibitory activity, with the raw sample exhibiting greatest inhibitory strength. Results suggest that different methods used (including changes in roasting temperature) influenced sample lipase inhibitory properties. Garzón et al. (2020) reported that BSG peptides demonstrated pancreatic lipase inhibitory activity following fractionation and purification using a range of chromatography methods [reversed phase high-performance liquid chromatography (RP-HPLC), gel filtration (FPLC) and anionic exchange]. Therefore, fluctuations in method conditions (fractionation/isolation process, sample preparation and method conditions such as enzyme volumes, etc.) could provide more valid results for the BSG samples.

Obesity encompasses behavioural, environmental, cultural, social, epigenetic and genetic characteristics (Tobore, 2020; Rodríguez-Pérez et al., 2017; Mopuri & Islam, 2017; Monteiro et al., 2017; Martel et al., 2017; Juul & Hemmingsson, 2015; Louzada et al., 2015; Sunkara & Verghese, 2014; Swinburn et al., 2004). The prevalence of obesity and weight gain is influenced by an increased sedentary life style and reduced physical activity but diet also plays a major role and with the easy availability of low cost energy dense foods/refined foods with elevated fat and simple sugar content and lower fibre content being a major contributing factor (Sunkara & Verghese, 2014; Kim & Park, 2011). Foods that influence adipocyte differentiation and increase lipolysis can help manage obesity with low-density foods further

assisting to conquer obesity by promoting satiety and diminishing energy intake (Sunkara & Verghese, 2014; Kim & Park, 2011). Plant derived phytochemicals have been shown to exhibit anti-obesogenic properties by a range of diverse physiological and cellular pathways, including the ability to decrease adipogenesis, suppress oxidative stress, stimulate mature adipocyte apoptosis and increase lipolysis (Cao et al., 2019; Rodríguez-Pérez et al., 2017; Martel et al., 2017; Mukherjee et al., 2015; Williams et al., 2013). Cao et al (2019) reported that the rich bioactive composition and fibre content of grains such as barley, wheat, sorghum, maize and buckwheat gives these grain crops their anti-obesogenic properties. Higher wholegrain consumption is associated with a lower risk of weight gain (Astrup et al., 2010). In this study the BSG fractions were demonstrated to inhibit triglyceride accumulation and promote an anti-adipogenic effect, which suggest that functional foods containing BSG-derived fractions could aid in weight management and lower the risk of the development of obesity, ultimately leading to increased mobility and better health.

4.4 Conclusion

This study is the first to report on the potential anti-obesity properties of BSG and BSG-derived fractions. It was evident that all of the BSG-derived fractions generated in this study can inhibit adipogenesis *in vitro,* with the most potent anti-adipogenic fractions being CIT-20 (water extracted phenolic fraction), CIT-17 (defatted fraction) and CIT-4 (soluble fraction). Future work however is needed to investigate the mechanism involved in inhibiting the differentiation process, which may be linked to suppression of relevant transcription factors or enzyme inhibition. Further *in vivo* studies would also be required to confirm any *in vitro* effects. None of the fractions inhibited pancreatic lipase based on the current investigations. These preliminary studies suggest that BSG and its components may have potential as food ingredients that could help in the design of functional foods to combat obesity.

Thesis Summary

BSG is a remarkable brewing industry co-product and a rich source of beneficial bioactive components and nutrients, making it a potentially desirable functional food ingredient (Lynch et al., 2016). Incorporating BSG and its components into innovative food solutions for older adults could help promote healthy ageing and ensures a greener environment by means of sustainable reuse of this abundant by-product (Aliyu & Bala, 2011).

The focus of the current research was to screen novel BSG-derived fractions generated by various extraction methods for *in vitro* bioactivities of interest, specifically antioxidant, anti-inflammatory and anti-obesity properties. Identifying more potent fractions with multiple bioactivities would be of interest to maximise the potential health benefits associated with BSG. A range of different phenolic fractions, protein hydrolysates, peptide-rich and fibre-rich fractions were generated and analysed. Table 5.1 summarises the bioactivities associated with all samples across all assays. Data from chemical-based antioxidant assays indicates that all fractions were associated with antioxidant properties, but the most potent fractions were CIT-2, CIT-4, CIT-14 and CIT-17 which were all protein-hydrolysates. The phenolic fractions and phenolic-derived fractions (CIT-3, CIT-7, CIT-8, CIT-9, CIT-11, CIT-12, CIT-13 and CIT-20) proved to have lower antioxidant properties than the protein fractions. However, the fibre fraction, CIT-15, demonstrated the lowest overall antioxidant activity, lower than both phenolic and protein fractions. Fraction defatting did not positively influence antioxidant properties, however CIT-17 demonstrated similar antioxidant values to the peptide-rich fraction (CIT-4), the fraction with overall greatest antioxidant activity. The simulated *in vitro* gastrointestinal digestion (SIGD) model proved to impact the antioxidant properties of BSG-derived fractions, nevertheless fraction combinations generated an antagonistic rather than the desired synergistic effect. However, other fraction combinations should be investigated, and it may be of interest to combine the fractions first before they are digested and investigated for bioactivity. According to Table 3.3, Table 3.4 and Table 5.1, CIT-21,

followed by fractions CIT-11 and CIT-14 had the greatest cellular antioxidant activity. All 21 BSG-derived fractions reduced TNFα secretion compared to the positive control, but not significantly, yet the protein hydrolysate (CIT-2) demonstrated the greatest immunomodulatory effect, followed by CIT-10 and CIT-12 (Table 3.5 and Table 5.1). The BSG intact sample and all 20 BSG-derived fractions exhibited anti-adipogenic properties *in vitro*, with CIT-20 demonstrating the greatest % decrease in adipocyte differentiation (triglyceride accumulation), followed by CIT-17 and CIT-4 (Table 4.3, Table 4.4 and Table 5.1). None of the fractions inhibited pancreatic lipase activity, however the precision of the data was poor and further studies are required to validate the results (Table 4.5 and Table 5.1).

Overall, based on these *in vitro* studies, BSG-derived fractions CIT-2, CIT-4, CIT-17 and CIT-20 were associated with more than one bioactivity, thus suggesting that these fractions should preferably be further investigated in combination studies and mechanistic studies to further support the potential to incorporate BSG as an bioactive ingredient in functional foods targeted at older adults.

Table 5.1: Bioactivity Summary of BSG-derived fractions (✓= Fraction possesses this bioactivity), (✓= Greatest/Strongest Bioactive Fraction), (**x** = No Bioactivity)

(* = Statistically Significant in comparison to Intact BSG)

CIT Code	Antioxidant Activity (Chemical Based)	Antioxidant Activity (Cellular Based)	Anti-inflammatory Properties	Anti-Adipogenic Properties	Lipase Inhibition
CIT-1	✓	✓	✓	✓	x
CIT-2	✓*	✓	✓	✓	x
CIT-3	✓	✓	✓	✓	x
CIT-4	✓*	✓	✓	✓*	x
CIT-5	✓	✓	✓	✓	x
CIT-6	✓	✓	✓	✓	x
CIT-7	✓	✓	✓	✓	x
CIT-8	✓	✓	✓	✓	x
CIT-9	✓	✓	✓	✓	x
CIT-10	✓	✓	✓	✓	x
CIT-11	✓	✓	✓	✓	x
CIT-12	✓	✓	✓	✓	x
CIT-13	✓	✓	✓	✓	x
CIT-14	✓*	✓*	✓	✓	x
CIT-15	✓	✓	✓	✓	x
CIT-16	✓	✓	✓	✓	x
CIT-17	✓*	✓	✓	✓*	x
CIT-18	✓	✓	✓	✓	x
CIT-19	✓	✓	✓	✓	x
CIT-20	✓	✓*	✓	✓*	x
CIT-21	✓	✓	✓	✓	x

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Appendices

ANOVA

TPC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5377.183	20	268.859	29.235	.000
Within Groups	386.248	42	9.196		
Total	5763.431	62			

p Value = 1.7669E-18

ANOVA

FRAP

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	265.659	20	13.283	58.273	.000
Within Groups	9.574	42	.228		
Total	275.232	62			

p Value = 2.469E-24

ANOVA

DPPH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	713.369	20	35.668	2.581	.005
Within Groups	580.448	42	13.820		
Total	1293.817	62			

p Value = 0.004797

TPC Assay

Significant differences between all BSG-derived Fractions (CIT-1-CIT-21) supporting Table 2.5, page 62 in the main body of the thesis

RED = Significant difference exists between BSG-derived fractions

	CIT-1	CIT-2	CIT-3	CIT-4	CIT-5	CIT-6	CIT-7	CIT-8	CIT-9	CIT-10	CIT-11	CIT-12	CIT-13	CIT-14	CIT-15	CIT-16	CIT-17	CIT-18	CIT-19	CIT-20	CIT-21
CIT-1		0.010	0.012	0.013	0.208	0.289	0.100	0.186	0.989	0.020	0.196	0.235	0.252	0.000	0.001	0.356	0.001	0.003	0.004	0.038	0.014
CIT-2	0.010		0.126	0.097	0.004	0.030	0.004	0.011	0.008	0.174	0.005	0.006	0.008	0.008	0.000	0.006	0.018	0.376	0.774	0.003	0.001
CIT-3	0.012	0.126		0.039	0.004	0.084	0.004	0.019	0.009	0.910	0.005	0.007	0.012	0.000	0.000	0.007	0.003	0.021	0.043	0.003	0.001
CIT-4	0.013	0.097	0.039		0.009	0.021	0.008	0.010	0.013	0.042	0.009	0.009	0.010	0.792	0.002	0.010	0.947	0.153	0.105	0.006	0.005
CIT-5	0.208	0.004	0.004	0.009		0.065	0.467	0.501	0.181	0.007	0.913	0.926	0.847	0.000	0.002	0.726	0.001	0.001	0.002	0.151	0.057
CIT-6	0.289	0.030	0.084	0.021	0.065		0.040	0.086	0.272	0.105	0.064	0.080	0.093	0.001	0.001	0.106	0.002	0.009	0.014	0.019	0.008
CIT-7	0.100	0.004	0.004	0.008	0.467	0.040		0.846	0.088	0.006	0.540	0.566	0.677	0.000	0.005	0.331	0.001	0.001	0.002	0.410	0.195
CIT-8	0.186	0.011	0.019	0.010	0.501	0.086	0.846		0.178	0.021	0.546	0.557	0.628	0.001	0.025	0.396	0.002	0.005	0.007	0.680	0.465
CIT-9	0.989	0.008	0.009	0.013	0.181	0.272	0.088	0.178		0.017	0.173	0.214	0.235	0.000	0.001	0.326	0.001	0.002	0.003	0.033	0.011
CIT-10	0.020	0.174	0.910	0.042	0.007	0.105	0.006	0.021	0.017		0.007	0.011	0.015	0.001	0.000	0.011	0.004	0.036	0.076	0.004	0.002
CIT-11	0.196	0.005	0.005	0.009	0.913	0.064	0.540	0.546	0.173	0.007		0.996	0.917	0.000	0.003	0.662	0.001	0.002	0.002	0.181	0.073
CIT-12	0.235	0.006	0.007	0.009	0.926	0.080	0.566	0.557	0.214	0.011	0.996		0.919	0.000	0.004	0.693	0.001	0.002	0.003	0.206	0.092
CIT-13	0.252	0.008	0.012	0.010	0.847	0.093	0.677	0.628	0.235	0.015	0.917	0.919		0.000	0.006	0.650	0.001	0.003	0.004	0.278	0.142
CIT-14	0.000	0.008	0.000	0.792	0.000	0.001	0.000	0.001	0.000	0.001	0.000	0.000	0.000		0.000	0.000	0.684	0.012	0.005	0.000	0.000
CIT-15	0.001	0.000	0.000	0.002	0.002	0.001	0.005	0.025	0.001	0.000	0.003	0.004	0.006	0.000		0.002	0.000	0.000	0.000	0.013	0.012
CIT-16	0.356	0.006	0.007	0.010	0.726	0.106	0.331	0.396	0.326	0.011	0.662	0.693	0.650	0.000	0.002		0.001	0.002	0.003	0.112	0.044
CIT-17	0.001	0.018	0.003	0.947	0.001	0.002	0.001	0.002	0.001	0.004	0.001	0.001	0.001	0.684	0.000	0.001		0.029	0.015	0.001	0.000
CIT-18	0.003	0.376	0.021	0.153	0.001	0.009	0.001	0.005	0.002	0.036	0.002	0.002	0.003	0.012	0.000	0.002	0.029		0.453	0.001	0.001
CIT-19	0.004	0.774	0.043	0.105	0.002	0.014	0.002	0.007	0.003	0.076	0.002	0.003	0.004	0.005	0.000	0.003	0.015	0.453		0.001	0.001
CIT-20	0.038	0.003	0.003	0.006	0.151	0.019	0.410	0.680	0.033	0.004	0.181	0.206	0.278	0.000	0.013	0.112	0.001	0.001	0.001		0.650
CIT-21	0.014	0.001	0.001	0.005	0.057	0.008	0.195	0.465	0.011	0.002	0.073	0.092	0.142	0.000	0.012	0.044	0.000	0.001	0.001	0.650	
Sig.	*	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t

FRAP Assay

Significant differences between all BSG-derived Fractions (CIT-1-CIT-21) supporting Table 2.5, page 62 in the main body of the thesis

RED = Significant difference exists between BSG-derived fractions

	CIT-1	CIT-2	CIT-3	CIT-4	CIT-5	CIT-6	CIT-7	CIT-8	CIT-9	CIT-10	CIT-11	CIT-12	CIT-13	CIT-14	CIT-15	CIT-16	CIT-17	CIT-18	CIT-19	CIT-20	CIT-21
CIT-1		0.000	0.000	0.000	0.081	0.002	0.002	0.003	0.001	0.003	0.001	0.000	0.001	0.000	0.036	0.391	0.000	0.001	0.002	0.329	0.067
CIT-2	0.000		0.051	0.022	0.000	0.015	0.008	0.002	0.033	0.000	0.003	0.083	0.005	0.014	0.000	0.000	0.232	0.001	0.001	0.000	0.000
CIT-3	0.000	0.051		0.301	0.000	0.080	0.037	0.006	0.316	0.001	0.008	0.668	0.020	0.181	0.000	0.000	0.121	0.002	0.001	0.000	0.000
CIT-4	0.000	0.022	0.301		0.000	0.199	0.094	0.012	0.820	0.001	0.023	0.189	0.063	0.801	0.000	0.000	0.034	0.004	0.002	0.000	0.000
CIT-5	0.081	0.000	0.000	0.000		0.004	0.004	0.008	0.001	0.009	0.001	0.000	0.001	0.000	0.004	0.019	0.000	0.002	0.004	0.421	0.864
CIT-6	0.002	0.015	0.080	0.199	0.004		0.717	0.145	0.311	0.026	0.429	0.061	0.774	0.224	0.001	0.001	0.023	0.088	0.045	0.003	0.003
CIT-7	0.002	0.008	0.037	0.094	0.004	0.717		0.208	0.171	0.030	0.667	0.029	0.892	0.102	0.001	0.001	0.011	0.122	0.056	0.003	0.003
CIT-8	0.003	0.002	0.006	0.012	0.008	0.145	0.208		0.028	0.159	0.246	0.005	0.124	0.011	0.001	0.002	0.002	0.840	0.373	0.007	0.006
CIT-9	0.001	0.033	0.316	0.820	0.001	0.311	0.171	0.028		0.005	0.065	0.218	0.146	0.955	0.000	0.000	0.063	0.013	0.008	0.001	0.001
CIT-10	0.003	0.000	0.001	0.001	0.009	0.026	0.030	0.159	0.005		0.016	0.001	0.011	0.001	0.000	0.001	0.000	0.097	0.350	0.009	0.005
CIT-11	0.001	0.003	0.008	0.023	0.001	0.429	0.667	0.246	0.065	0.016		0.007	0.484	0.021	0.000	0.000	0.002	0.106	0.036	0.001	0.001
CIT-12	0.000	0.083	0.668	0.189	0.000	0.061	0.029	0.005	0.218	0.001	0.007		0.016	0.113	0.000	0.000	0.246	0.001	0.001	0.000	0.000
CIT-13	0.001	0.005	0.020	0.063	0.001	0.774	0.892	0.124	0.146	0.011	0.484	0.016		0.063	0.000	0.000	0.005	0.052	0.021	0.001	0.001
CIT-14	0.000	0.014	0.181	0.801	0.000	0.224	0.102	0.011	0.955	0.001	0.021	0.113	0.063		0.000	0.000	0.017	0.003	0.001	0.000	0.000
CIT-15	0.036	0.000	0.000	0.000	0.004	0.001	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000		0.059	0.000	0.000	0.000	0.017	0.002
CIT-16	0.391	0.000	0.000	0.000	0.019	0.001	0.001	0.002	0.000	0.001	0.000	0.000	0.000	0.000	0.059		0.000	0.000	0.001	0.107	0.012
CIT-17	0.000	0.232	0.121	0.034	0.000	0.023	0.011	0.002	0.063	0.000	0.002	0.246	0.005	0.017	0.000	0.000		0.000	0.000	0.000	0.000
CIT-18	0.001	0.001	0.002	0.004	0.002	0.088	0.122	0.840	0.013	0.097	0.106	0.001	0.052	0.003	0.000	0.000	0.000		0.327	0.003	0.001
CIT-19	0.002	0.001	0.001	0.002	0.004	0.045	0.056	0.373	0.008	0.350	0.036	0.001	0.021	0.001	0.000	0.001	0.000	0.327		0.005	0.002
CIT-20	0.329	0.000	0.000	0.000	0.421	0.003	0.003	0.007	0.001	0.009	0.001	0.000	0.001	0.000	0.017	0.107	0.000	0.003	0.005		0.444
CIT-21	0.067	0.000	0.000	0.000	0.864	0.003	0.003	0.006	0.001	0.005	0.001	0.000	0.001	0.000	0.002	0.012	0.000	0.001	0.002	0.444	
Sig.	*	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t

DPPH Assay

Significant differences between all BSG-derived Fractions (CIT-1-CIT-21) supporting Table 2.5, page 62 in the main body of the thesis

RED = Significant difference exists between BSG-derived fractions

	CIT-1	CIT-2	CIT-3	CIT-4	CIT-5	CIT-6	CIT-7	CIT-8	CIT-9	CIT-10	CIT-11	CIT-12	CIT-13	CIT-14	CIT-15	CIT-16	CIT-17	CIT-18	CIT-19	CIT-20	CIT-21
CIT-1		0.000	0.001	0.000	0.257	0.014	0.005	0.015	0.067	0.037	0.004	0.943	0.008	0.001	0.880	0.242	0.414	0.002	0.010	0.200	0.517
CIT-2	0.000		0.006	0.451	0.003	0.001	0.006	0.013	0.000	0.064	0.005	0.266	0.009	0.156	0.000	0.000	0.393	0.010	0.009	0.000	0.000
CIT-3	0.001	0.006		0.008	0.022	0.032	0.320	0.317	0.001	0.658	0.327	0.469	0.359	0.035	0.003	0.004	0.790	0.936	0.294	0.001	0.003
CIT-4	0.000	0.451	0.008		0.003	0.002	0.006	0.011	0.000	0.048	0.006	0.239	0.009	0.096	0.001	0.001	0.339	0.011	0.009	0.000	0.001
CIT-5	0.257	0.003	0.022	0.003		0.200	0.057	0.104	0.938	0.137	0.049	0.877	0.070	0.006	0.248	0.753	0.598	0.024	0.087	0.078	0.492
CIT-6	0.014	0.001	0.032	0.002	0.200		0.166	0.361	0.029	0.382	0.134	0.680	0.224	0.004	0.019	0.056	0.851	0.039	0.299	0.007	0.035
CIT-7	0.005	0.006	0.320	0.006	0.057	0.166		0.801	0.007	0.937	0.940	0.544	0.962	0.021	0.007	0.015	0.926	0.324	0.824	0.003	0.011
CIT-8	0.015	0.013	0.317	0.011	0.104	0.361	0.801		0.033	0.814	0.751	0.573	0.845	0.036	0.017	0.041	0.974	0.315	0.965	0.008	0.029
CIT-9	0.067	0.000	0.001	0.000	0.938	0.029	0.007	0.033		0.082	0.004	0.886	0.014	0.000	0.094	0.639	0.579	0.002	0.021	0.018	0.285
CIT-10	0.037	0.064	0.658	0.048	0.137	0.382	0.937	0.814	0.082		0.968	0.540	0.917	0.146	0.038	0.079	0.908	0.639	0.833	0.019	0.058
CIT-11	0.004	0.005	0.327	0.006	0.049	0.134	0.940	0.751	0.004	0.968		0.538	0.908	0.018	0.006	0.012	0.915	0.332	0.769	0.003	0.009
CIT-12	0.943	0.266	0.469	0.239	0.877	0.680	0.544	0.573	0.886	0.540	0.538		0.549	0.325	0.926	0.923	0.657	0.464	0.567	0.780	0.984
CIT-13	0.008	0.009	0.359	0.009	0.070	0.224	0.962	0.845	0.014	0.917	0.908	0.549		0.030	0.010	0.023	0.934	0.356	0.871	0.005	0.016
CIT-14	0.001	0.156	0.035	0.096	0.006	0.004	0.021	0.036	0.000	0.146	0.018	0.325	0.030		0.001	0.001	0.509	0.051	0.028	0.000	0.001
CIT-15	0.880	0.000	0.003	0.001	0.248	0.019	0.007	0.017	0.094	0.038	0.006	0.926	0.010	0.001		0.244	0.401	0.003	0.013	0.283	0.478
CIT-16	0.242	0.000	0.004	0.001	0.753	0.056	0.015	0.041	0.639	0.079	0.012	0.923	0.023	0.001	0.244		0.542	0.005	0.030	0.055	0.595
CIT-17	0.414	0.393	0.790	0.339	0.598	0.851	0.926	0.974	0.579	0.908	0.915	0.657	0.934	0.509	0.401	0.542		0.781	0.965	0.293	0.481
CIT-18	0.002	0.010	0.936	0.011	0.024	0.039	0.324	0.315	0.002	0.639	0.332	0.464	0.356	0.051	0.003	0.005	0.781		0.295	0.001	0.004
CIT-19	0.010	0.009	0.294	0.009	0.087	0.299	0.824	0.965	0.021	0.833	0.769	0.567	0.871	0.028	0.013	0.030	0.965	0.295		0.006	0.021
CIT-20	0.200	0.000	0.001	0.000	0.078	0.007	0.003	0.008	0.018	0.019	0.003	0.780	0.005	0.000	0.283	0.055	0.293	0.001	0.006		0.108
CIT-21	0.517	0.000	0.003	0.001	0.492	0.035	0.011	0.029	0.285	0.058	0.009	0.984	0.016	0.001	0.478	0.595	0.481	0.004	0.021	0.108	
Sig.	*	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t

ANOVA

SOD - Negative Control Not Included

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	935.588	20	46.779	2.263	.013
Within Groups	868.173	42	20.671		
Total	1803.762	62			

p Value = 0.012864

ANOVA

SOD – Negative Control Included

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	935.723	21	44.558	2.172	.015
Within Groups	902.463	44	20.511		
Total	1838.186	65			

p Value = 0.015067

SOD Assay

Significant differences between all BSG-derived Fractions (CIT-1-CIT-21) supporting Table 3.4, page 91 in the main body of the thesis

RED = Significant difference exists between BSG-derived fractions

	CIT-1	CIT-2	CIT-3	CIT-4	CIT-5	CIT-6	CIT-7	CIT-8	CIT-9	CIT-10	CIT-11	CIT-12	CIT-13	CIT-14	CIT-15	CIT-16	CIT-17	CIT-18	CIT-19	CIT-20	CIT-21	Neg Con
CIT-1		0.243	0.213	0.843	0.744	0.543	0.811	0.242	0.644	0.297	0.104	0.261	0.076	0.032	0.472	0.106	0.166	0.064	0.250	0.022	0.102	0.226
CIT-2	0.243		0.880	0.195	0.195	0.527	0.206	0.975	0.178	0.589	0.427	0.488	0.839	0.509	0.350	0.752	0.863	0.947	0.853	0.271	0.945	0.745
CIT-3	0.213	0.880		0.157	0.167	0.564	0.177	0.905	0.153	0.638	0.335	0.496	0.981	0.323	0.319	0.860	0.991	0.756	0.964	0.157	0.897	0.840
CIT-4	0.843	0.195	0.157		0.858	0.441	0.940	0.193	0.738	0.200	0.088	0.139	0.034	0.019	0.284	0.050	0.111	0.034	0.190	0.015	0.060	0.155
CIT-5	0.744	0.195	0.167	0.858		0.417	0.925	0.193	0.883	0.221	0.089	0.192	0.068	0.030	0.326	0.089	0.131	0.057	0.196	0.021	0.085	0.174
CIT-6	0.543	0.527	0.564	0.441	0.417		0.448	0.538	0.371	0.821	0.206	0.904	0.469	0.152	0.835	0.557	0.536	0.342	0.607	0.084	0.435	0.655
CIT-7	0.811	0.206	0.177	0.940	0.925	0.448		0.205	0.810	0.236	0.092	0.203	0.067	0.029	0.355	0.090	0.137	0.057	0.208	0.021	0.086	0.184
CIT-8	0.242	0.975	0.905	0.193	0.193	0.538	0.205		0.177	0.603	0.407	0.497	0.868	0.473	0.352	0.776	0.890	0.911	0.877	0.248	0.976	0.767
CIT-9	0.644	0.178	0.153	0.738	0.883	0.371	0.810	0.177		0.200	0.084	0.176	0.068	0.030	0.287	0.087	0.122	0.057	0.178	0.021	0.081	0.159
CIT-10	0.297	0.589	0.638	0.200	0.221	0.821	0.236	0.603	0.200		0.212	0.838	0.470	0.112	0.498	0.612	0.598	0.311	0.693	0.060	0.447	0.764
CIT-11	0.104	0.427	0.335	0.088	0.089	0.206	0.092	0.407	0.084	0.212		0.173	0.279	0.664	0.133	0.252	0.313	0.370	0.331	0.975	0.333	0.269
CIT-12	0.261	0.488	0.496	0.139	0.192	0.904	0.203	0.497	0.176	0.838	0.173		0.146	0.047	0.434	0.273	0.420	0.124	0.562	0.031	0.244	0.589
CIT-13	0.076	0.839	0.981	0.034	0.068	0.469	0.067	0.868	0.068	0.470	0.279	0.146		0.128	0.048	0.700	0.990	0.515	0.970	0.065	0.791	0.783
CIT-14	0.032	0.509	0.323	0.019	0.030	0.152	0.029	0.473	0.030	0.112	0.664	0.047	0.128		0.027	0.106	0.257	0.303	0.326	0.436	0.259	0.195
CIT-15	0.472	0.350	0.319	0.284	0.326	0.835	0.355	0.352	0.287	0.498	0.133	0.434	0.048	0.027		0.095	0.241	0.057	0.378	0.021	0.121	0.351
CIT-16	0.106	0.752	0.860	0.05033	0.089	0.557	0.090	0.776	0.087	0.612	0.252	0.273	0.700	0.106	0.095		0.842	0.390	0.917	0.057	0.631	0.932
CIT-17	0.166	0.863	0.991	0.111	0.131	0.536	0.137	0.890	0.122	0.598	0.313	0.420	0.990	0.257	0.241	0.842		0.700	0.969	0.121	0.868	0.829
CIT-18	0.064	0.947	0.756	0.034	0.057	0.342	0.057	0.911	0.057	0.311	0.370	0.124	0.515	0.303	0.057	0.390	0.700		0.730	0.128	0.803	0.532
CIT-19	0.250	0.853	0.964	0.190	0.196	0.607	0.208	0.877	0.178	0.693	0.331	0.562	0.970	0.326	0.378	0.917	0.969	0.730		0.163	0.861	0.887
CIT-20	0.022	0.271	0.157	0.015	0.021	0.084	0.021	0.248	0.021	0.060	0.975	0.031	0.065	0.436	0.021	0.057	0.121	0.128	0.163		0.116	0.097
CIT-21	0.102	0.945	0.897	0.060	0.085	0.435	0.086	0.976	0.081	0.447	0.333	0.244	0.791	0.259	0.121	0.631	0.868	0.803	0.861	0.116		0.688
Sig.	*	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t	#

ANOVA

TNF-α – Negative & Positive Controls Not Included

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13.603	20	.680	.061	1.000
Within Groups	470.420	42	11.200		
Total	484.023	62			

ANOVA

TNF-α – Negative Control Included

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	17.585	21	.837	.077	1.000
Within Groups	481.019	44	10.932		
Total	498.604	65			

ANOVA

TNF-α – Positive Control Included

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	28.907	21	1.377	.117	1.000
Within Groups	517.275	44	11.756		
Total	546.182	65			

ANOVA

TNF-α – Negative & Positive Control Included

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	33.640	22	1.529	.133	1.000
Within Groups	527.874	46	11.476		
Total	561.514	68			

TNF-α Assay

Significant differences between all BSG-derived Fractions (CIT-1-CIT-21) supporting Table 3.5, page 94 in the main body of the thesis

RED = Significant difference exists between BSG-derived fractions

	CIT-1	CIT-2	CIT-3	CIT-4	CIT-5	CIT-6	CIT-7	CIT-8	CIT-9	CIT-10	CIT-11	CIT-12	CIT-13	CIT-14	CIT-15	CIT-16	CIT-17	CIT-18	CIT-19	CIT-20	CIT-21	Neg Con	Pos Con
CIT-1		0.678	0.846	0.911	0.924	0.807	0.852	0.896	0.940	0.773	0.993	0.853	0.987	0.809	0.742	0.950	0.991	0.978	0.867	0.979	0.970	0.611	0.497
CIT-2	0.678		0.597	0.787	0.652	0.582	0.818	0.615	0.764	0.923	0.705	0.879	0.731	0.607	0.497	0.605	0.674	0.722	0.630	0.700	0.717	0.919	0.349
CIT-3	0.846	0.597		0.781	0.924	0.955	0.729	0.944	0.806	0.671	0.846	0.742	0.879	0.942	0.914	0.877	0.854	0.835	0.989	0.877	0.894	0.548	0.632
CIT-4	0.911	0.787	0.781		0.851	0.749	0.952	0.822	0.974	0.870	0.923	0.935	0.915	0.756	0.683	0.859	0.904	0.937	0.804	0.901	0.899	0.720	0.465
CIT-5	0.924	0.652	0.924	0.851		0.883	0.798	0.977	0.877	0.732	0.921	0.803	0.948	0.876	0.833	0.962	0.932	0.909	0.939	0.950	0.964	0.597	0.568
CIT-6	0.807	0.582	0.955	0.749	0.883		0.701	0.900	0.772	0.649	0.809	0.714	0.843	0.984	0.965	0.835	0.815	0.799	0.947	0.838	0.857	0.537	0.680
CIT-7	0.852	0.818	0.729	0.952	0.798	0.701		0.765	0.925	0.908	0.869	0.974	0.869	0.715	0.626	0.791	0.846	0.885	0.756	0.851	0.854	0.744	0.425
CIT-8	0.896	0.615	0.944	0.822	0.977	0.900	0.765		0.849	0.699	0.894	0.777	0.926	0.892	0.849	0.933	0.904	0.881	0.958	0.926	0.942	0.559	0.575
CIT-9	0.940	0.764	0.806	0.974	0.877	0.772	0.925	0.849		0.846	0.950	0.913	0.938	0.777	0.709	0.891	0.933	0.964	0.827	0.927	0.923	0.700	0.483
CIT-10	0.773	0.923	0.671	0.870	0.732	0.649	0.908	0.699	0.846		0.792	0.948	0.802	0.666	0.574	0.713	0.768	0.808	0.699	0.780	0.788	0.851	0.397
CIT-11	0.993	0.705	0.846	0.923	0.921	0.809	0.869	0.894	0.950	0.792		0.866	0.981	0.810	0.747	0.945	0.985	0.986	0.866	0.974	0.965	0.642	0.505
CIT-12	0.853	0.879	0.742	0.935	0.803	0.714	0.974	0.777	0.913	0.948	0.866		0.864	0.722	0.653	0.806	0.847	0.879	0.763	0.849	0.850	0.818	0.453
CIT-13	0.987	0.731	0.879	0.915	0.948	0.843	0.869	0.926	0.938	0.802	0.981	0.864		0.839	0.793	0.975	0.994	0.969	0.895	0.995	0.986	0.677	0.550
CIT-14	0.809	0.607	0.942	0.756	0.876	0.984	0.715	0.892	0.777	0.666	0.810	0.722	0.839		0.986	0.835	0.816	0.801	0.934	0.836	0.852	0.567	0.714
CIT-15	0.742	0.497	0.914	0.683	0.833	0.965	0.626	0.849	0.709	0.574	0.747	0.653	0.793	0.986		0.767	0.751	0.737	0.907	0.784	0.808	0.451	0.686
CIT-16	0.950	0.605	0.877	0.859	0.962	0.835	0.791	0.933	0.891	0.713	0.945	0.806	0.975	0.835	0.767		0.960	0.930	0.898	0.979	0.992	0.538	0.506
CIT-17	0.991	0.674	0.854	0.904	0.932	0.815	0.846	0.904	0.933	0.768	0.985	0.847	0.994	0.816	0.751	0.960		0.970	0.874	0.987	0.977	0.609	0.503
CIT-18	0.978	0.722	0.835	0.937	0.909	0.799	0.885	0.881	0.964	0.808	0.986	0.879	0.969	0.801	0.737	0.930	0.970		0.855	0.961	0.953	0.659	0.499
CIT-19	0.867	0.630	0.989	0.804	0.939	0.947	0.756	0.958	0.827	0.699	0.866	0.763	0.895	0.934	0.907	0.898	0.874	0.855		0.894	0.909	0.582	0.634
CIT-20	0.979	0.700	0.877	0.901	0.950	0.838	0.851	0.926	0.927	0.780	0.974	0.849	0.995	0.836	0.784	0.979	0.987	0.961	0.894		0.990	0.642	0.534
CIT-21	0.970	0.717	0.894	0.899	0.964	0.857	0.854	0.942	0.923	0.788	0.965	0.850	0.986	0.852	0.808	0.992	0.977	0.953	0.909	0.990		0.664	0.561
Sig.	*	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t	#	+

ANOVA

Adipogenesis - Negative & Positive Controls Not Included

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.597	20	.180	1.544	.117
Within Groups	4.894	42	.117		
Total	8.491	62			

ANOVA

Adipogenesis - Negative Control Included

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.079	21	.289	2.601	.004
Within Groups	4.896	44	.111		
Total	10.975	65			

ANOVA

Adipogenesis - Positive Control Included

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8.956	21	.426	3.746	.000
Within Groups	5.009	44	.114		
Total	13.965	65			

p Value = 0.000110

ANOVA

Adipogenesis – Negative & Positive Control Included

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11.786	22	.536	4.917	.000
Within Groups	5.012	46	.109		
Total	16.798	68			

p Value = 0.000003

Adipogenesis Assay

Significant differences between all BSG-derived Fractions (CIT-1-CIT-21) supporting Table 4.4, page 117 in the main body of the thesis

RED = Significant difference exists between BSG-derived fractions

	CIT-1	CIT-2	CIT-3	CIT-4	CIT-5	CIT-6	CIT-7	CIT-8	CIT-9	CIT-10	CIT-11	CIT-12	CIT-13	CIT-14	CIT-15	CIT-16	CIT-17	CIT-18	CIT-19	CIT-20	CIT-21	Neg Con	Pos Con
CIT-1		0.084	0.993	0.008	0.465	0.805	0.320	0.016	0.379	0.475	0.716	0.590	0.028	0.976	0.320	0.471	0.030	0.343	0.952	0.042	0.821	0.000	0.001
CIT-2	0.084		0.577	0.434	0.250	0.295	0.138	0.009	0.083	0.191	0.441	0.141	0.013	0.362	0.159	0.267	0.296	0.958	0.473	0.283	0.287	0.001	0.001
CIT-3	0.993	0.577		0.434	0.862	0.926	0.530	0.157	0.770	0.677	0.777	0.844	0.312	0.994	0.480	0.579	0.348	0.629	0.967	0.325	0.917	0.046	0.014
CIT-4	0.008	0.434	0.434		0.052	0.112	0.089	0.005	0.029	0.118	0.348	0.057	0.006	0.202	0.112	0.203	0.542	0.632	0.299	0.484	0.157	0.000	0.000
CIT-5	0.465	0.250	0.862	0.052		0.845	0.254	0.015	0.251	0.370	0.627	0.398	0.026	0.757	0.263	0.402	0.072	0.520	0.856	0.084	0.621	0.000	0.001
CIT-6	0.805	0.295	0.926	0.112	0.845		0.313	0.026	0.417	0.452	0.676	0.563	0.056	0.872	0.309	0.447	0.101	0.489	0.949	0.105	0.742	0.002	0.001
CIT-7	0.320	0.138	0.530	0.089	0.254	0.313		0.304	0.557	0.778	0.816	0.498	0.688	0.419	0.872	0.963	0.075	0.194	0.420	0.074	0.480	0.008	0.014
CIT-8	0.016	0.009	0.157	0.005	0.015	0.026	0.304		0.051	0.180	0.354	0.049	0.306	0.059	0.468	0.481	0.006	0.024	0.075	0.007	0.070	0.001	0.015
CIT-9	0.379	0.083	0.770	0.029	0.251	0.417	0.557	0.051		0.792	0.909	0.846	0.139	0.648	0.501	0.649	0.035	0.216	0.638	0.040	0.771	0.001	0.002
CIT-10	0.475	0.191	0.677	0.118	0.370	0.452	0.778	0.180	0.792		0.970	0.707	0.438	0.581	0.681	0.794	0.098	0.266	0.571	0.095	0.663	0.008	0.009
CIT-11	0.716	0.441	0.777	0.348	0.627	0.676	0.816	0.354	0.909	0.970		0.852	0.610	0.747	0.734	0.813	0.290	0.479	0.726	0.275	0.807	0.059	0.040
CIT-12	0.590	0.141	0.844	0.057	0.398	0.563	0.498	0.049	0.846	0.707	0.852		0.130	0.768	0.455	0.600	0.057	0.284	0.740	0.061	0.894	0.001	0.002
CIT-13	0.028	0.013	0.312	0.006	0.026	0.056	0.688	0.306	0.139	0.438	0.610	0.130		0.141	0.886	0.823	0.008	0.047	0.170	0.010	0.172	0.000	0.004
CIT-14	0.976	0.362	0.994	0.202	0.757	0.872	0.419	0.059	0.648	0.581	0.747	0.768	0.141		0.389	0.519	0.158	0.482	0.947	0.152	0.888	0.007	0.003
CIT-15	0.320	0.159	0.480	0.112	0.263	0.309	0.872	0.468	0.501	0.681	0.734	0.455	0.886	0.389		0.931	0.094	0.201	0.389	0.091	0.439	0.014	0.029
CIT-16	0.471	0.267	0.579	0.203	0.402	0.447	0.963	0.481	0.649	0.794	0.813	0.600	0.823	0.519	0.931		0.170	0.305	0.509	0.161	0.571	0.033	0.045
CIT-17	0.030	0.296	0.348	0.542	0.072	0.101	0.075	0.006	0.035	0.098	0.290	0.057	0.008	0.158	0.094	0.170		0.448	0.229	0.873	0.126	0.006	0.001
CIT-18	0.343	0.958	0.629	0.632	0.520	0.489	0.194	0.024	0.216	0.266	0.479	0.284	0.047	0.482	0.201	0.305	0.448		0.566	0.408	0.405	0.013	0.002
CIT-19	0.952	0.473	0.967	0.299	0.856	0.949	0.420	0.075	0.638	0.571	0.726	0.740	0.170	0.947	0.389	0.509	0.229	0.566		0.216	0.847	0.014	0.005
CIT-20	0.042	0.283	0.325	0.484	0.084	0.105	0.074	0.007	0.040	0.095	0.275	0.061	0.010	0.152	0.091	0.161	0.873	0.408	0.216		0.123	0.014	0.001
CIT-21	0.821	0.287	0.917	0.157	0.621	0.742	0.480	0.070	0.771	0.663	0.807	0.894	0.172	0.888	0.439	0.571	0.126	0.405	0.847	0.123		0.006	0.003
Sig.	*	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t	#	+

ANOVA

Lipase Inhibition

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10202.502	20	510.125	.440	.975
Within Groups	48651.518	42	1158.369		
Total	58854.020	62			

Lipase Inhibition Assay

Significant differences between all BSG-derived Fractions (CIT-1-CIT-21) supporting Table 4.5, page 119 in the main body of the thesis

RED = Significant difference exists between BSG-derived fractions

	CIT-1	CIT-2	CIT-3	CIT-4	CIT-5	CIT-6	CIT-7	CIT-8	CIT-9	CIT-10	CIT-11	CIT-12	CIT-13	CIT-14	CIT-15	CIT-16	CIT-17	CIT-18	CIT-19	CIT-20	CIT-21
CIT-1		0.939	0.418	0.844	0.621	0.999	0.915	0.774	0.723	0.440	0.973	0.911	0.868	0.847	0.708	0.626	0.529	0.600	0.487	0.326	0.234
CIT-2	0.939		0.479	0.911	0.680	0.940	0.970	0.838	0.789	0.509	0.965	0.979	0.936	0.787	0.666	0.594	0.590	0.661	0.552	0.380	0.281
CIT-3	0.418	0.479		0.535	0.809	0.415	0.546	0.618	0.661	0.916	0.438	0.459	0.498	0.267	0.245	0.253	0.893	0.818	0.902	0.848	0.685
CIT-4	0.844	0.911	0.535		0.748	0.844	0.951	0.920	0.868	0.572	0.871	0.924	0.970	0.672	0.570	0.519	0.652	0.728	0.615	0.425	0.314
CIT-5	0.621	0.680	0.809	0.748		0.620	0.731	0.825	0.870	0.871	0.643	0.676	0.716	0.477	0.418	0.393	0.915	0.987	0.896	0.681	0.548
CIT-6	0.999	0.940	0.415	0.844	0.620		0.915	0.773	0.722	0.436	0.974	0.911	0.868	0.843	0.704	0.622	0.527	0.598	0.485	0.323	0.231
CIT-7	0.915	0.970	0.546	0.951	0.731	0.915		0.883	0.837	0.582	0.938	0.986	0.975	0.785	0.677	0.605	0.647	0.714	0.617	0.447	0.346
CIT-8	0.774	0.838	0.618	0.920	0.825	0.773	0.883		0.950	0.664	0.799	0.845	0.889	0.611	0.525	0.482	0.732	0.808	0.701	0.501	0.382
CIT-9	0.723	0.789	0.661	0.868	0.870	0.722	0.837	0.950		0.712	0.748	0.791	0.836	0.558	0.481	0.447	0.777	0.853	0.747	0.538	0.413
CIT-10	0.440	0.509	0.916	0.572	0.871	0.436	0.582	0.664	0.712		0.462	0.485	0.530	0.261	0.243	0.257	0.964	0.882	0.979	0.756	0.587
CIT-11	0.973	0.965	0.438	0.871	0.643	0.974	0.938	0.799	0.748	0.462		0.939	0.896	0.817	0.685	0.608	0.550	0.622	0.509	0.343	0.248
CIT-12	0.911	0.979	0.459	0.924	0.676	0.911	0.986	0.845	0.791	0.485	0.939		0.952	0.729	0.609	0.550	0.579	0.655	0.535	0.356	0.254
CIT-13	0.868	0.936	0.498	0.970	0.716	0.868	0.975	0.889	0.836	0.530	0.896	0.952		0.686	0.577	0.525	0.618	0.695	0.578	0.390	0.282
CIT-14	0.847	0.787	0.267	0.672	0.477	0.843	0.785	0.611	0.558	0.261	0.817	0.729	0.686		0.781	0.674	0.381	0.451	0.324	0.195	0.124
CIT-15	0.708	0.666	0.245	0.570	0.418	0.704	0.677	0.525	0.481	0.243	0.685	0.609	0.577	0.781		0.842	0.338	0.395	0.292	0.184	0.124
CIT-16	0.626	0.594	0.253	0.519	0.393	0.622	0.605	0.482	0.447	0.257	0.608	0.550	0.525	0.674	0.842		0.328	0.375	0.293	0.199	0.146
CIT-17	0.529	0.590	0.893	0.652	0.915	0.527	0.647	0.732	0.777	0.964	0.550	0.579	0.618	0.381	0.338	0.328		0.926	0.985	0.755	0.609
CIT-18	0.600	0.661	0.818	0.728	0.987	0.598	0.714	0.808	0.853	0.882	0.622	0.655	0.695	0.451	0.395	0.375	0.926		0.907	0.686	0.549
CIT-19	0.487	0.552	0.902	0.615	0.896	0.485	0.617	0.701	0.747	0.979	0.509	0.535	0.578	0.324	0.292	0.293	0.985	0.907		0.753	0.597
CIT-20	0.326	0.380	0.848	0.425	0.681	0.323	0.447	0.501	0.538	0.756	0.343	0.356	0.390	0.195	0.184	0.199	0.755	0.686	0.753		0.833
CIT-21	0.234	0.281	0.685	0.314	0.548	0.231	0.346	0.382	0.413	0.587	0.248	0.254	0.282	0.124	0.124	0.146	0.609	0.549	0.597	0.833	
Sig.	*	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t

Complete U937 cell line MTT results for BSG fractions CIT-1-CIT-21 supporting Table 3.2A, page 86 in the main body of the thesis

CIT 1 - BSG Intact Digestate – U937 Cell Line

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	124.8583	99.73073	108.305	101.4314	96.35771	57.24206
N=2	100	68.82556	67.29035	65.91825	64.834	44.15659	66.83938
Average	**100**	**96.84192**	**83.51054**	**87.11162**	**83.13271**	**70.25715**	**62.04072**
Standard Deviation	**0**	**39.62111**	**22.93881**	**29.97195**	**25.87827**	**36.91176**	**6.786326**

CIT 2 - Protein MTL Digestate – U937 Cell Line

	0%	0.50%	1%	2%	2.50%	5%	10%
N=1	100	122.2605	126.7433	142.5287	134.9808	155.1341	245.4789
N=2	100	133.285	138.2609	130.1449	120.9179	142.7536	224.2029
N=3	100	101.4732	95.625	85.3125	87.90179	96.02679	139.0179
Average	**100**	**119.0063**	**120.2097**	**119.3287**	**114.6002**	**131.3048**	**202.8999**
Standard Deviation	**0**	**16.15365**	**22.05607**	**30.10261**	**24.16701**	**31.1725**	**56.33696**

CIT 3 - New Phenolic Fraction Digestate – U937 Cell Line

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	71.07430775	66.15843	67.89345	70.40834	59.30599	51.6211
N=2	100	95.16733116	95.90431	92.34022	91.10789	184.8858	89.89972
Average	**100**	**83.12081945**	**81.03137**	**80.11683**	**80.75812**	**122.0959**	**70.76041**
Standard Deviation	**0**	**17.03634023**	**21.03352**	**17.28648**	**14.63679**	**88.79835**	**27.06707**

CIT 4 - Soluble Fraction –U937 Cells

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	41.84579	61.02482	157.6134	58.91693	53.73735	158.4038
N=2	100	63.02844	123.5204	54.41968	37.35588	22.82859	11.68332
Average	**100**	**52.43712**	**92.2726**	**106.0165**	**48.13641**	**38.28297**	**85.04357**
Standard Deviation	**0**	**14.97839**	**44.19102**	**72.96896**	**15.24597**	**21.85579**	**103.7471**

CIT 5 - BSG pre-digested with CHOases +Alc/Fla (Insoluble Fraction) –U937 Cells

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	82.95371	77.81043	77.22263	61.42542	44.08523	4.188097
N=2	100	101.502	85.92885	83.00395	74.07115	55.81028	17.62846
Average	**100**	**92.22784**	**81.86964**	**80.11329**	**67.74828**	**49.94775**	**10.90828**
Standard Deviation	**0**	**13.1156**	**5.74059**	**4.088012**	**8.941877**	**8.290859**	**9.503771**

CIT 6 - Phenolic MTL Digestate – U937 Cell Line

	0%	0.50%	1%	2%	2.50%	5%	10%
N=1	100	52.1969	34.00784	3.598022	3.63781	0.642301	1.097027
N=2	100	58.80361	13.43115	3.781038	1.015801	0.1693	0.790068
N=3	100	60.34676	39.59732	6.375839	3.076063	0.167785	1.118568
Average	**100**	**57.11575**	**29.0121**	**4.584966**	**2.576558**	**0.326462**	**1.001888**
Standard Deviation	**0**	**4.329168**	**13.77988**	**1.553638**	**1.380529**	**0.273525**	**0.183757**

CIT 7 - Permeate 3kDa Fraction –U937 Cells

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	98.81657	93.153	77.51479	70.83686	46.83009	5.91716
N=2	100	83.565	70.56419	65.49469	55.27392	45.46198	3.434178
Average	**100**	**91.19079**	**81.85859**	**71.50474**	**63.05539**	**46.14604**	**4.675669**
Standard Deviation	**0**	**10.78448**	**15.9727**	**8.4995**	**11.00466**	**0.967403**	**1.755733**

CIT 8 - Permeate 10kDa Fraction –U937 Cells

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	85.6779661	73.55932	65.25424	56.44068	47.45763	15.84746
N=2	100	90.0893582	83.91552	76.19821	67.7498	48.90333	15.67831
Average	**100**	**87.8836622**	**78.73742**	**70.72623**	**62.09524**	**48.18048**	**15.76288**
Standard Deviation	**0**	**3.1193253**	**7.322935**	**7.738559**	**7.996755**	**1.022267**	**0.119605**

CIT 9 - Permeate 50kDa Fraction –U937 Cells

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	93.2072227	89.93981	71.19518	67.41187	46.94755	3.697334
N=2	100	87.6635514	81.96262	68.2243	65.14019	50.46729	3.271028
Average	**100**	**90.4353871**	**85.95121**	**69.70974**	**66.27603**	**48.70742**	**3.484181**
Standard Deviation	**0**	**3.91996757**	**5.640728**	**2.100733**	**1.60632**	**2.488832**	**0.301444**

CIT 10 - Not Separated Fraction –U937 Cells

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	125.4934	111.431	125.6642	88.2358	109.9103	69.02179
N=2	100	118.9292	103.2329	156.8754	142.3201	99.8976	64.64307
Average	**100**	**122.2113**	**107.3319**	**141.2698**	**115.2779**	**104.9039**	**66.83243**
Standard Deviation	**0**	**4.641577**	**5.796952**	**22.06959**	**38.24336**	**7.080044**	**3.096222**

CIT 11 - Retentate 3kDa Digestate - U937 cell line

	0%	0.10%	0.20%	0.30%	0.40%	0.50%	1%	2%	2.50%	5%	10.00%	20.00%
N=1	100	98.5218	92.60902	75.83149	61.86253	106.4302	98.37398	97.85661	97.11752	26.38581	3.399852	3.325942
N=2	100	98.4917	87.33032	76.77225	61.2368	95.55053	97.20965	93.74057	91.70437	30.46757	3.167421	2.941176
Average	**100**	**98.50675**	**89.96967**	**76.30187**	**61.54967**	**100.9903**	**97.79182**	**95.79859**	**94.41095**	**28.42669**	**3.283636**	**3.133559**
Standard Deviation	**0**	**0.021283**	**3.732605**	**0.665219**	**0.442455**	**7.693058**	**0.823306**	**2.910481**	**3.82767**	**2.886242**	**0.164354**	**0.272071**

CIT 12 - Retentate 10kDa Fraction –U937 Cells

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	74.32778	76.50448	66.38924	57.17029	21.06274	0.576184
N=2	100	118.7905	104.5356	92.11663	81.74946	56.04752	1.403888
Average	**100**	**96.55914**	**90.52006**	**79.25294**	**69.45988**	**38.55513**	**0.990036**
Standard Deviation	**0**	**31.43989**	**19.82102**	**18.19201**	**17.38009**	**24.73797**	**0.585275**

CIT 13 - Retentate 50kDa Fraction –U937 Cells

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	90.46875	72.96875	61.64063	47.89063	32.26563	5.07813

CIT 14 - Peptide Fraction –U937 Cells

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	98.03545	114.9483	114.4018	257.3264	97.57755	156.7208
N=2	100	97.66234	96.36364	85.06494	95.97403	61.81818	46.36364
Average	**100**	**97.84889**	**105.656**	**99.73335**	**176.6502**	**79.69786**	**101.5422**
Standard Deviation	**0**	**0.263831**	**13.14134**	**20.74428**	**114.0934**	**25.28569**	**78.03432**

CIT 15 - Old Fibre Fraction –U937 Cells

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	77.7998	91.27849	84.44004	63.72646	45.58969	1.090188
N=2	100	94.74104	84.30279	72.27092	60	37.21116	2.310757
Average	**100**	**86.27042**	**87.79064**	**78.35548**	**61.86323**	**41.40042**	**1.700473**
Standard Deviation	**0**	**11.97926**	**4.932568**	**8.60487**	**2.635006**	**5.924521**	**0.863072**

CIT 16 - Fibre New Fraction –U937 Cells

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	122.9155	103.1877	67.25645	54.51289	50.16476	29.67765
N=2	100	85.46864	98.97624	84.22622	144.0513	96.75977	58.19501
Average	**100**	**104.1921**	**101.082**	**75.74133**	**99.28209**	**73.46226**	**43.93633**
Standard Deviation	**0**	**26.47891**	**2.977934**	**11.99944**	**63.3132**	**32.94765**	**20.16482**

CIT 17 - Defatted d1 Fraction –U937 Cells

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	123.1119199	114.8316652	102.7298	87.35214	54.95905	18.65332
N=2	100	91.01321586	71.54185022	59.20705	66.78414	46.78414	14.36123
Average	**100**	**107.0625679**	**93.18675769**	**80.9684**	**77.06814**	**50.8716**	**16.50728**
Standard Deviation	**0**	**22.69721131**	**30.61052169**	**30.7752**	**14.54377**	**5.780536**	**3.034964**

CIT 18 - Defatted d2 Fraction –U937 Cells

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	93.4833204	89.44919	77.88984	70.20946	36.69511	7.137316
N=2	100	98.47619048	84.66667	81.2381	72.85714	40.7619	24.57143
Average	**100**	**95.97975544**	**87.05793**	**79.56397**	**71.5333**	**38.72851**	**15.85437**
Standard Deviation	**0**	**3.530492286**	**3.381751**	**2.367576**	**1.872191**	**2.875656**	**12.32778**

CIT 19 - BSG Hydrolysate da Digestate - U937 cell line

	0%	0.50%	1.00%	1.50%	2.00%	2.50%	5%	10%	20%
N=1	100	108.4871	104.674	96.30996	89.05289	47.10947	3.874539	3.198032	2.275523
N=2	100	107.1341	102.46	90.1599	84.80935	44.64945	5.904059	5.596556	3.075031
Average	100	107.8106	103.567	93.23493	86.93112	45.87946	4.889299	4.397294	2.675277
Standard Deviation	0	0.956725	1.56555	4.34875	3.000638	1.7395	1.435088	1.696013	0.565338

CIT 20 - H2O Extraction Digestate - U937 cell line

	0%	0.10%	0.20%	0.30%	0.40%	0.50%	1%	2%	2.50%	5%	10.00%	20.00%
N=1	100	96.59173	84.62654	66.27991	49.96374	93.40102	89.63017	88.25236	83.39376	35.46048	21.7549	24.2204

CIT 21 - Aqueous Phenolic Extraction Fraction –U937 Cells

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	151.7181	112.3348	104.4053	94.27313	61.32159	27.57709
N=2	100	105.7167	103.9768	81.27589	72.16239	54.92958	13.83596
Average	100	128.7174	108.1558	92.84059	83.21776	58.12558	20.70652
Standard Deviation	0	32.52791	5.909998	16.35495	15.63466	4.519833	9.71645

Complete Jurkat T cell line MTT results for BSG fractions CIT-1-CIT-21 supporting Table 3.2B, page 87 in the main body of the thesis

CIT 1 - BSG Intact Digestate - Jurkat Cell Line

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	103.2289	102.4691	86.98955	76.35328	67.4264	57.64482
N=2	100	107.5975	105.1335	98.25462	93.32649	80.1848	56.57084
Average	**100**	**105.4132**	**103.8013**	**92.62209**	**84.83988**	**73.8056**	**57.10783**
Standard Deviation	**0**	**3.089113**	**1.883969**	**7.965605**	**12.00187**	**9.021554**	**0.75942**

CIT 2 - Protein MTL Digestate - Jurkat Cell Line

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	72.16117	85.62271	72.52747	66.57509	51.19048	45.2381
N=2	100	98.99749	95.11278	94.11028	91.4787	71.55388	54.88722
Average	**100**	**85.57933**	**90.36775**	**83.31887**	**79.02689**	**61.37218**	**50.06266**
Standard Deviation	**0**	**18.97614**	**6.710494**	**15.26135**	**17.60951**	**14.3991**	**6.82296**

CIT 3 - New Phenolic fraction Digestate - Jurkat Cell Line

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	151.0811	152.4324	144.5946	126.7568	94.32432	74.86486
N=2	100	110.1464	109.205	106.2762	102.1967	77.92887	22.90795
Average	**100**	**130.6138**	**130.8187**	**125.4354**	**114.4767**	**86.1266**	**48.88641**
Standard Deviation	**0**	**28.94516**	**30.5664**	**27.09523**	**17.36662**	**11.59334**	**36.73909**

CIT 4 - Soluble Digestate - Jurkat Cell Line

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	100.885	110.9145	112.3894	110.0295	190.2655	148.9676
N=2	100	91.54176	88.65096	88.97216	134.7966	65.41756	50.6424
Average	**100**	**96.21336**	**99.78271**	**100.6808**	**122.413**	**127.8415**	**99.80497**
Standard Deviation	**0**	**6.60664**	**15.74267**	**16.55847**	**17.51297**	**88.28082**	**69.52638**

CIT 5 - Insoluble Digestate - Jurkat Cell Line

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	109.405	109.309	94.91363	84.45298	70.6334	54.7025
N=2	100	92.71357	95.14238	80.65327	70.43551	59.21273	44.89112
Average	**100**	**101.0593**	**102.2257**	**87.78345**	**77.44424**	**64.92306**	**49.79681**
Standard Deviation	**0**	**11.80262**	**10.01733**	**10.0836**	**9.911844**	**8.075631**	**6.937688**

CIT 6 - Phenolic MTL Digestate - Jurkat Cell Line

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	95.20137	87.48929	81.74807	78.23479	72.75064	50.47129
N=2	100	94.04553	88.2662	80.38529	76.44483	69.26445	49.56217
Average	**100**	**94.62345**	**87.87774**	**81.06668**	**77.33981**	**71.00755**	**50.01673**
Standard Deviation	**0**	**0.8173**	**0.549359**	**0.963633**	**1.26569**	**2.465112**	**0.642847**

CIT 7 - Permeate 3kda Digestate - Jurkat Cell Line

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	81.69935	76.09711	78.89823	68.25397	50.51354	24.27638
N=2	100	104.3912	89.42116	87.12575	77.44511	69.36128	50.3992
Average	**100**	**93.04528**	**82.75913**	**83.01199**	**72.84954**	**59.93741**	**37.33779**
Standard Deviation	**0**	**16.04558**	**9.421528**	**5.817737**	**6.499119**	**13.32736**	**18.47163**

CIT 8 - Permeate 10kda Digestate - Jurkat Cell Line

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	102.1127	101.4085	88.02817	75.35211	55.33199	42.35412
N=2	100	101.4218	102.0853	98.00948	84.92891	69.57346	56.68246
Average	**100**	**101.7672**	**101.7469**	**93.01882**	**80.14051**	**62.45273**	**49.51829**
Standard Deviation	**0**	**0.488522**	**0.47861**	**7.057852**	**6.771818**	**10.07024**	**10.13167**

CIT 9 - Permeate 50Kda Digestate - Jurkat Cell Line

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	121.3251	107.4534	104.1408	89.64803	77.84679	67.49482
N=2	100	93.36898	96.63102	78.71658	74.70588	70.69519	46.25668
Average	**100**	**107.347**	**102.0422**	**91.42868**	**82.17696**	**74.27099**	**56.87575**
Standard Deviation	**0**	**19.76793**	**7.652593**	**17.97763**	**10.5657**	**5.056947**	**15.01763**

CIT 10 - Not Separated Digestate - Jurkat Cell Line

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	204.7872	99.20213	335.1064	37.23404	36.96809	10.90426
N=2	100	184.375	112.5	262.5	93.75	87.5	93.75
Average	**100**	**194.5811**	**105.8511**	**298.8032**	**65.49202**	**62.23404**	**52.32713**
Standard Deviation	**0**	**14.43363**	**9.403016**	**51.34047**	**39.96282**	**35.73146**	**58.58079**

CIT 11 - Retentate 3kDa Digestate - Jurkat Cell Line

	0%	0.10%	0.20%	0.30%	0.40%	0.50%	1%	2%	2.50%	5%	10.00%	20.00%
N=1	100	93.08855	82.77538	73.97408	76.1879	90.44276	93.7905	106.1555	111.2851	61.60907	19.81641	2.537797
N=2	100	95.85586	84.2042	68.34835	70.45045	95.01502	98.37838	111.2312	105.9459	49.84985	16.51652	2.582583
Average	**100**	**94.4722**	**83.48979**	**71.16122**	**73.31918**	**92.72889**	**96.08444**	**108.6934**	**108.6155**	**55.72946**	**18.16647**	**2.56019**
Standard Deviation	**0**	**1.956779**	**1.010333**	**3.977994**	**4.056993**	**3.233069**	**3.244122**	**3.589079**	**3.77535**	**8.315025**	**2.33338**	**0.031668**

CIT 12 - Retentate 10kda Digestate - Jurkat Cell Line

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	109.3333	111.7333	79.46667	69.51111	59.2	49.51111
N=2	100	105.1121	105.7399	89.77578	74.08072	63.67713	47.44395
Average	**100**	**107.2227**	**108.7366**	**84.62123**	**71.79591**	**61.43857**	**48.47753**
Standard Deviation	**0**	**2.984857**	**4.23799**	**7.289647**	**3.2312**	**3.165809**	**1.461706**

CIT 13 - Retentate 50kDa Digestate - Jurkat Cell Line

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	92.7026618	93.19419	74.3043	65.07108	47.38355	20.22188
N=2	100	93.7571219	90.51766	83.76201	69.65652	51.73368	28.30107
Average	**100**	**93.2298919**	**91.85593**	**79.03315**	**67.3638**	**49.55861**	**24.26147**
Standard Deviation	**0**	**0.74561589**	**1.892593**	**6.687611**	**3.242393**	**3.07601**	**5.71285**

CIT 14 - Peptide Digestate - Jurkat Cell Line

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	76.64234	73.23601	85.40146	73.47932	136.983	101.4599
N=2	100	82.283	77.05113	102.497	70.98692	61.95006	133.7693
Average	**100**	**79.46267**	**75.14357**	**93.94924**	**72.23312**	**99.46651**	**117.6146**
Standard Deviation	**0**	**3.988549**	**2.697697**	**12.08839**	**1.762392**	**53.05628**	**22.84624**

CIT 15 - Fibre Old Digestate - Jurkat Cell Line

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	95.33214	87.43268	80.34111	76.57092	60.41293	36.80431
N=2	100	100.4647	99.07063	92.47212	74.53532	63.19703	49.53532
Average	**100**	**97.89841**	**93.25165**	**86.40662**	**75.55312**	**61.80498**	**43.16981**
Standard Deviation	**0**	**3.629259**	**8.229278**	**8.577917**	**1.439386**	**1.968656**	**9.002182**

CIT 16 - Fibre New Digestate - Jurkat Cell Line

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	80.11794	86.35215	79.69671	68.3235	55.77085	37.65796
N=2	100	113.8542	111.5625	103.125	89.6875	75.83333	60.9375
Average	**100**	**96.98606**	**98.95732**	**91.41086**	**79.0055**	**65.80209**	**49.29773**
Standard Deviation	**0**	**23.85511**	**17.82641**	**16.5663**	**15.10663**	**14.18632**	**16.46112**

CIT 17 - Defatted d1 Digestate - Jurkat Cell Line

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	99.08497	124.9673	111.2418	96.86275	43.66013	-8.10458
N=2	100	105.9351	98.7682	91.60134	85.44233	70.21277	34.60246
Average	**100**	**102.51**	**111.8678**	**101.4216**	**91.15254**	**56.93645**	**13.24894**
Standard Deviation	**0**	**4.84374**	**18.52558**	**13.88792**	**8.075454**	**18.77555**	**30.19844**

CIT 18 - Defatted d2 Digestate - Jurkat Cell Line

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	94.59459	100	94.59459	105.4054	102.7027	118.9189
N=2	100	97.4359	87.17949	84.61538	87.17949	-112.821	41.02564
Average	**100**	**96.01525**	**93.58974**	**89.60499**	**96.29245**	**-5.05891**	**79.97228**
Standard Deviation	**0**	**2.009105**	**9.065472**	**7.056367**	**12.88767**	**152.3979**	**55.07887**

CIT 19 - BSG Hydrolysate da Digestate - Jurkat Cell Line

	0%	0.10%	0.20%	0.30%	0.40%	0.50%	1%	2%	2.50%	5%	10.00%	20.00%
N=1	100	86.8646	86.50472	74.04408	73.95412	62.21323	81.60144	85.33513	82.861	50.60729	17.94872	3.643725
N=2	100	88.48539	80.07086	67.09477	60.85031	80.6023	83.30381	87.4225	86.80248	55.62445	21.21346	3.720106
Average	**100**	**87.67499**	**83.28779**	**70.56943**	**67.40221**	**71.40776**	**82.45262**	**86.37882**	**84.83174**	**53.11587**	**19.58109**	**3.681915**
Standard Deviation	**0**	**1.14607**	**4.549429**	**4.913905**	**9.26579**	**13.00304**	**1.203757**	**1.47599**	**2.787048**	**3.547667**	**2.308524**	**0.05401**

CIT 20 - H2O Extraction Digestate - Jurkat Cell Line

	0%	0.10%	0.20%	0.30%	0.40%	0.50%	1%	2%	2.50%	5%	10.00%	20.00%
N=1	100	102.5119617	95.87321	83.37321	73.20574	100.8373	105.6818	95.69378	79.12679	66.38756	24.46172	0.478469
N=2	100	88.914417	85.41068	74.21022	65.13498	91.38426	96.49627	91.09707	98.73636	54.79609	22.86043	0.229753
Average	**100**	**95.71318936**	**90.64194**	**78.79171**	**69.17036**	**96.11079**	**101.089**	**93.39543**	**88.93158**	**60.59183**	**23.66107**	**0.354111**
Standard Deviation	**0**	**9.61491608**	**7.39812**	**6.479207**	**5.70689**	**6.684322**	**6.495166**	**3.250364**	**13.86606**	**8.196404**	**1.132288**	**0.175869**

CIT 21 - Aqueous Phenolic Extraction Digestate - Jurkat Cell Line

	0%	0.50%	1%	2%	2.50%	3.50%	5%
N=1	100	87.4822665	88.25002	82.6838	73.35392	60.26871	45.15883
N=2	100	100.746818	101.5357	82.03429	80.5091	73.1461	46.08478
Average	**100**	**94.1145423**	**94.89287**	**82.35905**	**76.93151**	**66.70741**	**45.6218**
Standard Deviation	**0**	**9.37945438**	**9.394401**	**0.459274**	**5.059477**	**9.105689**	**0.654745**

Complete 3T3 cell line MTT results for BSG fractions CIT-1-CIT-21 supporting Table 4.2, page 112 in the main body of the thesis

CIT 1 - BSG Intact Digestate - 3T3 Cell Line

	0%	0.50%	1.00%	2.00%	2.50%	5.00%	10%
N=1	100	82.83909	81.52649	69.85902	62.37239	23.86971	26.05737
N=2	100	70.4698	72.55034	52.01342	25.50336	8.120805	19.26174
Average	**100**	**76.65444**	**77.03842**	**60.93622**	**43.93787**	**15.99526**	**22.65956**
Standard Deviation	**0**	**8.746407**	**6.347103**	**12.61874**	**26.07034**	**11.13616**	**4.805229**

CIT 2 -Protein MTL Digestate –3T3 Cell Line

	0%	0.50%	1.00%	2.00%
N=1	100	113.5017	105.0523	88.85017
N=2	100	125.3252	115.438	82.39376
Average	**100**	**119.4135**	**110.2451**	**85.62196**
Standard Deviation	**0**	**8.360474**	**7.343815**	**4.565378**

CIT 3 -New Phenolic fraction Digestate - 3T3 Cell Line

	0%	0.50%	1.00%	2.00%
N=1	100	91.42582	92.19653	65.99229
N=2	100	84.28571	98.83929	94.55357
Average	**100**	**87.85577**	**95.51791**	**80.27293**
Standard Deviation	**0**	**5.048816**	**4.697136**	**20.19587**

CIT 4 -Soluble Digestate - 3T3 Cell Line

	0%	0.50%	1.00%	2.00%	2.50%	5.00%	10%
N=1	100	94.19908	85.56361	50.42848	61.37113	27.09295	14.10679
N=2	100	64.42953	95.4698	67.95302	40.26846	8.053691	17.28188
Average	**100**	**79.3143**	**90.51671**	**59.19075**	**50.81979**	**17.57332**	**15.69433**
Standard Deviation	**0**	**21.05025**	**7.004731**	**12.39172**	**14.92184**	**13.46279**	**2.245127**

CIT 5 -Insoluble Digestate -3T3 Cell Line

	0%	0.50%	1.00%	2.00%	2.50%	5.00%	10%
N=1	100	105.6673	97.50152	75.19805	72.15113	18.82998	69.95734
N=2	100	106.4302	138.8027	72.50554	81.15299	19.73392	63.85809
Average	**100**	**106.0487**	**118.1521**	**73.8518**	**76.65206**	**19.28195**	**66.90772**
Standard Deviation	**0**	**0.539437**	**29.20431**	**1.90389**	**6.36528**	**0.639184**	**4.312821**

CIT 6 –Phenolic MTL Digestate -3T3 Cell Line

	0%	0.50%	1%	2%	2.50%	5%	10%
N=1	100	80.12048	63.95582	32.32932	13.45382	31.4257	25
N=2	100	64.94983	70.43478	5.41806	6.488294	22.14047	28.82943
Average	**100**	**72.53516**	**67.1953**	**18.87369**	**9.971055**	**26.78309**	**26.91472**
Standard Deviation	**0**	**10.72727**	**4.581316**	**19.02913**	**4.925367**	**6.565652**	**2.707817**

CIT 7 -Permeate 3kda Digestate -3T3 Cell Line

	0%	0.50%	1.00%	2.00%	2.50%	5.00%	10%
N=1	100	104.1073	80.01095	90.52574	72.28916	30.06572	38.1161
N=2	100	75.30788	88.05419	79.18719	74.69212	35.52956	74.44581
N=3	100	90.36017	91.52542	84.2161	78.49576	58.84534	75.68856
Average	**100**	**89.92513**	**86.53019**	**84.64301**	**75.15901**	**41.4802**	**62.75016**
Standard Deviation	**0**	**14.40466**	**5.90658**	**5.681316**	**3.129534**	**15.28477**	**21.34277**

CIT 8 – Permeate 10kda Digestate -3T3 Cell Line

	0%	0.50%	1.00%	2.00%	2.50%	5.00%	10%
N=1	100	112.8997	85.50165	82.80044	73.043	18.13671	38.53363
N=2	100	71.87621	71.06383	65.26112	49.78723	34.89362	58.18182
Average	**100**	**92.38794**	**78.28274**	**74.03078**	**61.41512**	**26.51517**	**48.35772**
Standard Deviation	**0**	**29.00797**	**10.20908**	**12.40217**	**16.44431**	**11.84892**	**13.89337**

CIT 9 -Permeate 50Kda Digestate - 3T3 Cell Line

	0%	0.50%	1.00%	2.00%	2.50%	5.00%	10%
N=1	100	93.71824	89.51501	61.01617	61.06236	23.60277	32.194
N=2	100	90.03129	77.73804	67.09879	59.54403	27.04515	52.88333
Average	**100**	**91.87477**	**83.62653**	**64.05748**	**60.30319**	**25.32396**	**42.53866**
Standard Deviation	**0**	**2.607069**	**8.327575**	**4.301067**	**1.073617**	**2.434129**	**14.62957**

CIT 10 - Not Separated Digestate –3T3 Cell Line

	0%	0.50%	1.00%	2.00%
N=1	100	137.0995	169.4772	126.6442
N=2	100	84.60821	83.11567	71.17537
Average	**100**	**110.8539**	**126.2965**	**98.90978**
Standard Deviation	**0**	**37.11694**	**61.06685**	**39.22237**

CIT 11 - Retentate 3kDa Digestate -3T3 Cell Line

	0%	0.10%	0.20%	0.30%	0.40%	0.50%	1%	2%	2.50%	5%	10.00%	20.00%
N=1	100	75.17241	63.96552	50.45977	50.91954	75.97701	74.94253	69.5977	79.94253	42.41379	56.32184	18.85057
N=2	100	82.53761	64.09418	55.72269	57.09614	85.80772	85.87312	80.83715	62.19751	37.01766	54.54545	16.21975
Average	**100**	**78.85501**	**64.02985**	**53.09123**	**54.00784**	**80.89236**	**80.40782**	**75.21742**	**71.07002**	**39.71573**	**55.43365**	**17.53516**
Standard Deviation	**0**	**5.207978**	**0.090978**	**3.72145**	**4.367516**	**6.951359**	**7.729095**	**7.947489**	**12.54762**	**3.815643**	**1.256094**	**1.860273**

CIT 12 - Retentate 10kda Digestate –3T3 Cell Line

	0%	0.50%	1.00%	2.00%
N=1	100	97.41935	89.67742	77.2043
N=2	100	88.22906	107.4231	98.72747
Average	**100**	**92.82421**	**98.55027**	**87.96588**
Standard Deviation	**0**	**6.498522**	**12.5481**	**15.21918**

CIT 13 - Retentate 50kDa Digestate -3T3 Cell Line

	0%	0.50%	1.00%	2.00%	2.50%	5.00%	10%
N=1	100	70.50265	85.84656	68.78307	72.22222	7.407407	12.10317
N=2	100	61.66924	88.5626	29.36631	41.26739	36.01236	8.500773
Average	**100**	**66.08594**	**87.20458**	**49.07469**	**56.74481**	**21.70989**	**10.30197**
Standard Deviation	**0**	**6.246159**	**1.920527**	**27.87186**	**21.88837**	**20.22676**	**2.547283**

CIT 14 - Peptide Digestate - 3T3 Cell Line

	0%	0.50%	1.00%	2.00%
N=1	100	97.78457	88.69366	65.69901
N=2	100	103.2283	90.55118	73.54331
Average	**100**	**100.5065**	**89.62242**	**69.62116**
Standard Deviation	**0**	**3.849332**	**1.313466**	**5.546758**

CIT 15 - Fibre Old Digestate - 3T3 Cell Line

	0%	0.50%	1.00%	2.00%
N=1	100	96.49123	79.25697	95.45924
N=2	100	83.26271	99.04661	94.17373
Average	**100**	**89.87697**	**89.15179**	**94.81648**
Standard Deviation	**0**	**9.353974**	**13.99339**	**0.908991**

CIT 16 - Fibre New Digestate –3T3 Cell Line

	0%	0.50%	1.00%	2.00%	2.50%	5.00%	10%
N=1	100	99.19893	86.31509	67.28972	60.34713	16.75567	21.76235
N=2	100	72.49344	87.1916	77.37533	74.80315	53.75328	76.95538
Average	**100**	**85.84619**	**86.75334**	**72.33252**	**67.57514**	**35.25448**	**49.35887**
Standard Deviation	**0**	**18.88364**	**0.619789**	**7.131602**	**10.22195**	**26.16126**	**39.02737**

CIT 17 - Defatted d1 Digestate -3T3 Cell Line

	0%	0.50%	1.00%	2.00%	2.50%	5.00%	10%
N=1	100	117.8795	96.86324	60.47679	42.72271	6.900878	21.7064
N=2	100	108.3763	88.33763	50	59.14948	37.95103	38.2732
Average	**100**	**113.1279**	**92.60043**	**55.23839**	**50.9361**	**22.42595**	**29.9898**
Standard Deviation	**0**	**6.719819**	**6.028515**	**7.408208**	**11.61548**	**21.95577**	**11.71449**

CIT 18 - Defatted d2 Digestate – 3T3 Cell Line

	0%	0.50%	1.00%	2.00%
N=1	100	98.56902	107.7441	76.85185
N=2	100	116.2521	106.5506	78.60697
Average	**100**	**107.4105**	**107.1473**	**77.72941**
Standard Deviation	**0**	**12.5038**	**0.843951**	**1.241053**

CIT 19 - BSG Hydrolysate da Digestate - 3T3 Cell Line

	0%	0.10%	0.20%	0.30%	0.40%	0.50%	1%	2%	2.50%	5%	10.00%	20.00%
N=1	100	62.7237	67.91794	41.33566	43.78001	76.16761	72.32649	61.58883	52.90266	31.25273	19.42383	2.79354
N=2	100	84.77752	63.13817	56.81499	41.96721	69.13349	67.54098	55.50351	47.58782	23.09133	26.60422	4.637002
Average	**100**	**73.75061**	**65.52806**	**49.07532**	**42.87361**	**72.65055**	**69.93374**	**58.54617**	**50.24524**	**27.17203**	**23.01402**	**3.715271**
Standard Deviation	**0**	**15.5944**	**3.379805**	**10.94554**	**1.28184**	**4.973876**	**3.383868**	**4.302966**	**3.75816**	**5.770976**	**5.077298**	**1.303525**

CIT 20 - H2O Extraction Digestate - 3T3 Cell Line

	0%	0.10%	0.20%	0.30%	0.40%	0.50%	1%	2%	2.50%	5%	10.00%	20.00%
N=1	100	76.11174	67.67389	56.21437	60.8894	87.11517	91.73318	77.93615	102.2235	59.92018	55.75827	13.39795
N=2	100	75.47281	72.16312	52.77778	34.10165	81.974	72.51773	72.69504	73.34515	52.54137	27.60047	17.7896
Average	**100**	**75.79228**	**69.9185**	**54.49607**	**47.49553**	**84.54458**	**82.12546**	**75.31559**	**87.78432**	**56.23078**	**41.67937**	**15.59377**
Standard Deviation	**0**	**0.451793**	**3.174367**	**2.430036**	**18.94179**	**3.635356**	**13.58738**	**3.706025**	**20.42007**	**5.217607**	**19.91057**	**3.105366**

CIT 21 - Aqueous Phenolic Extraction Digestate - 3T3 Cell Line

	0%	0.50%	1.00%	2.00%
N=1	100	90.00861	91.0422	90.78381
N=2	100	98.35616	84.74886	72.69406
Average	**100**	**94.18239**	**87.89553**	**81.73894**
Standard Deviation	**0**	**5.90261**	**4.450068**	**12.79138**

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