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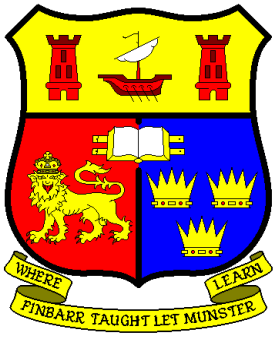
UCC
University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

The National University of Ireland

Cork

School of Food and Nutritional Sciences

Supervisors: Professor N.M. O'Brien and Dr. J.P. Kerry



Cellular and *In-vitro* Models to Assess Antioxidant Activities of Seaweed Extracts and the Potential Use of the Extracts as Ingredients

Thesis presented by

Anthoney Michael Naim O'Sullivan, B.Sc.

For the degree of

Doctor of Philosophy in Food and Nutritional Sciences

January, 2013

Table of Contents

Acknowledgements iii
Publications v
Abstract ix

Chapter 1 1
Literature review

Chapter 2 57
Antioxidant activities assessed using in-vitro and cellular model systems of five brown seaweeds harvested in spring from the west coast of Ireland.

Chapter 3 85
Assessment of the ability of seaweed extracts to protect against hydrogen peroxide and *tert*-butyl hydroperoxide induced cellular damage in Caco-2 cells.

Chapter 4 101
The effect of solvents on the antioxidant activity in Caco-2 cells of Irish brown seaweed extracts prepared using accelerated solvent extraction (ASE®).

Chapter 5 125
An examination of the potential of seaweed extracts as functional ingredients in milk.

Chapter 6 162
An investigation of the performance of seaweed extracts as potential functional ingredients in yogurt.

Chapter 7 204
Additional study: Assessment of the antioxidant potential of extracts of Chlorella, Spirulina and Kelp food supplements in Caco-2 cells.

Chapter 8 225
General discussion

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Publications

Papers:

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2. O'Sullivan, A.M., O'Callaghan, Y.C., O'Grady, M.N., Queguineur, B., Hanniffy, D., Troy, D.J., Kerry, J.P. & O'Brien, N.M. (2012). Assessment of the ability of seaweed extracts to protect against hydrogen peroxide and *tert*-butyl hydroperoxide induced cellular damage in Caco-2 cells. *Food Chemistry, 134*, 1137-1140.
3. O'Sullivan, A.M., O'Callaghan, Y.C., O'Grady, M.N., Hayes, M., Kerry, J.P. & O'Brien, N.M. (2013). Use of a Caco-2 cell model to assess the antioxidant activity of Irish brown seaweed extracts prepared using accelerated solvent extraction (ASE®). *Journal of Functional Foods*. (DOI number: 10.1016/j.jff.2013.02.007).
4. O'Sullivan, A.M., O'Callaghan, Y.C., O'Connor, T.P. & O'Brien, N.M. (2013). Comparison of the antioxidant activity of commercial honeys, before and after *in-vitro* digestion. *Polish Journal of Food and Nutrition Sciences*. (DOI number: 10.2478/v10222-012-0080-6).
5. O'Sullivan, A.M., O'Callaghan, Y.C., O'Grady, M.N., Smyth, T., O'Brien, N.M, & Kerry, J.P. (2013). An Examination of the Potential of Seaweed Extracts as Functional Ingredients in Milk. *International Journal of Dairy Technology*. (Submitted).

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Abstracts

1. O'Sullivan, A.M., O'Callaghan, Y.C., O'Grady, M.N., Kraan, S., Kerry, J.P. & O'Brien, N.M. (2009). Determination of the antioxidant potential of seaweed extracts using different methods. *Proceedings of the Nutrition Society, 68, OCE3*, E110.
2. O'Sullivan, A.M., O'Callaghan, Y.C., O'Grady, M.N., Kerry, J.P. & O'Brien, N.M. (2010). The antioxidant activity of five brown seaweeds, sourced from the west coast of Ireland, assessed in the Caco-2 cell line. *Proceedings of the Nutrition Society, 69, OCE6*, E403.
3. O'Sullivan, A.M., O'Callaghan, Y.C., O'Connor, T. P, & O'Brien, N.M. (2011). The content and bioaccessibility of carotenoids from selected commercially available health supplements. *Proceedings of the Nutrition Society, 70, OCE3*, E62.
4. O'Sullivan, A.M., O'Callaghan, Y.C., O'Grady, M.N., Hayes, M., Kerry, J.P. & O'Brien, N.M. (2010). The influence of the extraction method on the DNA protective effects of seaweed extracts in Caco-2 cells. *Proceedings of the Nutrition Society, 70, OCE3*, E77.
5. O'Sullivan, A.M., O'Callaghan, Y.C., O'Connor, T. P, & O'Brien, N.M. (2011). Bioaccessibility of polyphenols and carotenoids from wild Irish berries subjected to an *in-vitro* digestion procedure. *Proceedings of the Nutrition Society, 70, OCE3*, E61.
6. O'Sullivan, A.M., O'Callaghan, Y.C., O'Grady, M.N., Queguineur, B., Hanniffy, D., Troy, D.J., Kerry, J.P. & O'Brien, N.M. (2011).The ability of seaweed extracts to protect against oxidative damage to DNA in Caco-2 cells. *Proceedings of the 40th Annual UCC Food Research Conference,* A31.

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8. O'Callaghan, Y.C., O'Sullivan, A.M., O'Connor, T. P, & O'Brien, N.M. (2012). The effect of an *in-vitro* digestion on the antioxidant activity of a range of honeys. *Proceedings of the 41st Annual UCC Food Research Conference,* P12.

Abstract

Seaweeds contain a range of antioxidant compounds such as polyphenols, carotenoids, sulphated polysaccharides and vitamins and have the potential to be used as ingredients in neutraceuticals. The antioxidant activity of crude 60% methanol extracts prepared from five Irish seaweeds, *Ascophyllum nodosum, Laminaria hyperborea, Pelvetia canaliculata, Fucus vesiculosus* and *Fucus serratus* were examined using *in-vitro* assays and a cell model system to determine the antioxidant activity of the extracts and their ability to protect against H_2O_2 and *tert*-BOOH-induced DNA damage and alterations in cellular antioxidant status in the human adenocarcinoma, Caco-2 cell line.

To optimise the extraction of antioxidant compounds from seaweeds, an accelerated solvent extraction (ASE®) was used in combination with food grade solvents. The antioxidant activity of these extracts against H_2O_2 and *tert*-BOOH-induced DNA damage and alterations in cellular antioxidant status was also assessed.

Extracts that exhibited the highest antioxidant activity, *A. nodosum* (100% water and 80% ethanol extracts) and *F. vesiculosus* (60% ethanol extract) were selected as ingredients for incorporation into fluid milk and yogurt at concentrations of 0.25% and 0.5%. The addition of the seaweed extracts to milk and yogurt did not affect the pH or shelf-life properties of the products. Seaweed addition did however significantly influence the colour properties of the milk and yogurt. Yellowness values were significantly higher in yogurts containing *F. vesiculosus* at both concentrations and *A. nodosum* (80% ethanol) at the 0.5% concentration. In milk, the *F. vesiculosus* (60% ethanol) and *A. nodosum* (80% ethanol) at both the 0.25% and the 0.5% concentrations had higher greenness and yellowness values than the milk containing *A. nodosum* (100% water).

Sensory analysis revealed that appearance and flavour governed the overall acceptability of yogurts with the control yogurt, and yogurts containing *A. nodosum* (100% water) were the most preferred samples by panellists. However, in the milk trial the perception of a fishy taste was the determining factor in the negative perception of milk. The unsupplemented control and the milk containing *A. nodosum* (100% water) at a concentration of 0.5% were the most overall accepted milk samples by the sensory panellists.

The antioxidant activity of the extracts in milk and yogurt remained stable during storage as determined by the *in-vitro* assays. Seaweed supplemented milk

and yogurt were also subjected to an *in-vitro* digestion procedure which mimics the human digestive system. The milk and yogurt samples and their digestates were added to Caco-2 cells to investigate their antioxidant potential however neither the undigested or digested samples protected against H_2O_2-induced DNA damage in Caco-2 cells.

1. Introduction

Algae are a group of chlorophyll containing, oxygen-producing, photosynthetic organisms which are genetically diverse and inhabit a wide range of aquatic environments. Algae are classified as either microalgae or macroalgae. Microalgae are small (µm) unicellular organisms that can exist freely or in groups and include the commercially important *Chlorella vulgaris* and *Dunaliella salina*. Macroalgae, or seaweed as they are commonly known, are large multicellular organisms that generally inhabit marine waters, although green macroalgae are also found in fresh water habitats. Seaweed is divided into three classes depending on the colour of the algae; brown (Phaeophyceae), green (Chlorophyta) and red (Rhodophyta) consisting of approximately 1,800, 1,200 and 6,000 species, respectively.

Marine products have been identified as a potential source of bioactive compounds in recent years. The main sources investigated include seaweed and by-products from the fish, crustacean and mollusc processing industries. A wide range of antioxidant, antimicrobial, anticancer, and other health promoting compounds have been extracted from marine products using different extraction techniques. The health benefits of these extracts have been demonstrated in cell culture based models as well as in both human and animal trials. In addition to their health promoting effects some marine derived compounds have exhibited potential for use as food ingredients due to their antimicrobial and antioxidant properties (Rajauria et al., 2012). Seaweed consumption has been linked to a variety of health benefits such as a reduced incidence of cancer (Yang et al., 2010; Alekseyenko et al., 2007; Hiqashi-Okaj et al., 1999; Hoshiyama et al., 1993), diabetes (Lee et al., 2010) and heart disease (Bocanegra et al., 2008). Seaweed has been a part of the staple diet in many Far East countries such as Korea, Japan and China for millennia, and is used in soups, salads, sushi, deserts and many other dishes. The red algae *Chondrus crispus* has been used to produce a blancmange-like desert in Ireland and Scotland and *Porphyra yezoensis* has been used to produce laverbread, a savoury dish, in Wales. Seaweed has also had limited culinary applications in other coastal parts of Europe.

Historically, seaweeds have also had many medicinal applications. *Laminaria* (brown algae) powder has been used to successfully treat goitre since the 4th century in China and was later adopted for the same purpose in 19th century

Europe (Kelly, 1961). Iodine was previously commercially extracted from seaweed, however this practise is no longer deemed to be economically viable. Seaweeds from the *Laminaria*, *Ecklonia*, *Porphyra* and *Sargassum* genera are commonly used in Chinese medicine to treat a wide variety of human ailments including tumours, edema, testicular pain and throat infections (Dharmananda, 2002). Apart from their extensive culinary and medicinal use in the Far East, seaweeds are cultured and harvested worldwide for the production of phycocolloids such as agars, carrageenans and alginates which are widely used as gelling, thickening and emulsifying agents in the food and pharmaceutical industries (Glickman, 1987; Zemke-White & Ohno, 1999). Agar is also extensively used as a growth medium for bacterial, fungal and plant cells.

The present review will focus on the antioxidant compounds found in seaweed and the various extraction techniques employed to isolate these compounds. The main categories of plant derived antioxidants identified in seaweeds are carotenoids, phenolics, alkaloids (Herraiz & Galisteo, 2003) and organosulphur compounds (Sahu, 2002). To date, research in the area of seaweed antioxidants has focused primarily on polyphenol compounds and carotenoids. Marine-derived extracts are particularly rich in polyphenols and carotenoids and have previously exhibited antioxidant activity in a number of studies. The profile of antioxidant compounds in algae differ depending on the colour of the species; for instance the antioxidant pigments α-carotene, β-carotene, lutein and zeaxanthin are common in both red and green algae while brown seaweed contains β-carotene and fucoxanthin. Fucoidan (or heterofucan), a cell wall component with potent antioxidant activity, is exclusively found in brown seaweeds. Algae are also a source of tocopherols; α-, β-, and γ-tocopherol are present in brown algae while green and red algae contain only α-tocopherol (Burtin, 2003).

2. Antioxidant constituents of marine algae

An antioxidant can be defined as any substance, that when present at low concentrations compared with those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate (Halliwell, 1993). Compounds such as polyphenols, carotenoids and fucoidan are currently extracted from algae for commercial purposes. The technology also exists to commercially extract antioxidants such as vitamin E from algae however most antioxidant compounds are

extracted from other sources (vitamin E from edible oils) or industrially synthesised (vitamin C). The predominant antioxidants in seaweeds are in the categories of pigments, polyphenols and fucoidan.

2.1. Pigments (carotenoids and other pigments)

Seaweeds contain a diverse range of carotenoids unlike higher plants which contain a uniform profile of a small number of carotenoids (Demmig-Adams et al., 1996). The characteristic colour of brown algae is due to the dominant xanthophyll, fucoxanthin, which masks other pigments including chlorophylls and carotenoids. The dominance of the phycobiliprotein pigments phycoerythrin and phycocyanin give red algae their distinctive colour. The dominance of chlorophyll a and b gives green seaweed its colour. In plants, carotenoids serve two primary functions, in photosynthesis, to collect light and pass it to chlorophyll and to protect against light-induced damage to chlorophyll and other organelles. Chlorophyll a absorbs the majority of light (in green and brown algae) and is assisted by other chlorophylls b, c, d and f and pigments such as fucoxanthin, siphonaxanthin, and peridinin, which absorb light at different wavelengths to that of chlorophyll a. Phycobiliproteins are the main light-absorbing pigments in red algae absorbing light in the range 520 nm to 630 nm which allows red algae to inhabit deep water. Absorbed light is subsequently used in the photosynthesis process to produce energy and other components. Carotenoids such as violaxanthin, antheraxanthin and zeaxanthin also protect seaweed by dissipating potentially harmful photo energy via the xanthophyll cycle (Demmig-Adams et al., 1996).

2.2. Polyphenols

This term encompasses several thousand plant-based secondary metabolites which have a typical polyphenol structure containing several hydroxyl groups on aromatic rings (Manach *et al*., 2005). Polyphenols can be classified into different groups based on the number of phenol rings they contain and how these rings are bound to one another. For example the structure of one such polyphenol group, the flavonoids, consisting of 2 aromatic rings (A and B) that are bound together by 3 carbon atoms that form an oxygenated heterocycle (ring C). Polyphenols are highly-structured molecules consisting of multiple phenol groups. Seaweeds are considered a superior source of polyphenols in comparison to terrestrial plants because

terrestrial plants contain polyphenols with a lower number of rings in the structure and are derived from gallic and ellagic acid (Figure 2), while seaweed polyphenols are multiple-ringed (up to eight) derived from phloroglucinol units (Figure 3) (1,3,5-trihydroxybenzine) (Burtin, 2003). Polyphenols containing a higher number of rings in their structure exhibit greater antioxidant activity (Gupta & Abu-Ghannam, 2011). Polyphenols are predominantly found in brown seaweeds and phlorotannins (multi-ringed phloroglucinol-based tannins), such as the well studied eckol (Figure 4), are exclusively found in brown algae. Other polyphenols such as bromophenol are present in all classes of seaweed. Phlorotannins can account for up to 40% of the dry weight of some brown seaweed (Arnold et al., 1995). The polyphenol profile is highly dependent on the colour of the seaweed. Yoshie-Stark et al. (2003) examined the polyphenol profile of a number of Japanese seaweed species and found that the flavonoids morin, hesperidin and catechol were found in most green, red and brown seaweed while caffeic acid was found mostly in red species. Rutin was generally absent from brown species while quercitrin was exclusively found in brown species. Polyphenols have a number of roles in seaweed. Firstly they act as antioxidant agents in the seaweed, protecting them from oxidative stress. In addition, phlorotannins have been documented to act as an herbivore deterrent and this function is postulated to be due to their GIT irritating and nutrient reducing properties (Koivikko, 2008). Studies have found that phlorotannin levels increase in seaweeds in response to grazing by herbivores which reduces subsequent grazing (Rohde et al., 2004; Coleman et al., 2007). Other studies dispute the role of phlorotannins as anti-herbivore agents (Jormalainen et al., 2001; Toth & Pavia, 2002) and it has been suggested that phlorotannins may only be used to deter herbivores by certain species of seaweed (Targett & Arnold, 1998). Other studies have suggested that polyphenols may act as anti-fouling agents and prevent the colonisation of seaweed by invertebrates (such as barnacles), algae, bacteria and other organisms which reduce the productivity of the host seaweed (Wikström & Pavia, 2004; Lau & Qian, 2000). Koivikko (2008) suggest that phlorotannins have an important role in the structural integrity of algal cell walls and in reproduction.

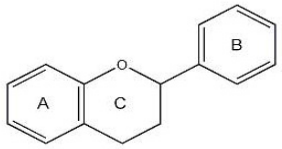


Figure 1. Structure of a typical flavonoid consisting of A, B and C ring.

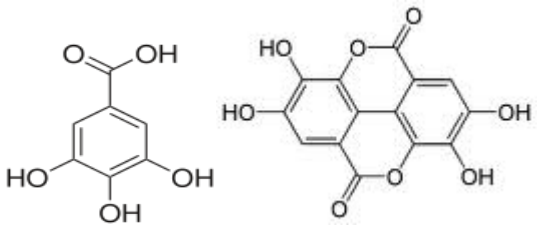


Figure 2. Structures of gallic acid and ellagic acid.

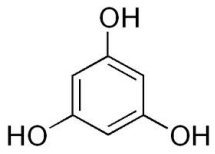


Figure 3. Structure of phloroglucinol.

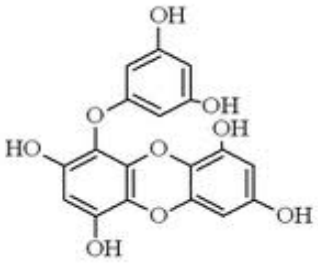


Figure 4. Structure of eckol.

2.3. Antioxidant polysaccharides

Seaweed is rich source of both sulphated and non-sulphated polysaccharides. Sulphated-polysaccharides are structural components that protect the surface of seaweed from drying out and contribute to the gel structure of the cell wall (Percival and McDowell, 1967). Non-sulphated polysaccharides include laminarin and alginic acid (alginate) which function as energy storage and structural components, respectively. Sulphated-polysaccharides are the most investigated type of polysaccharide. The sulphated-polysaccharide profile is highly dependent on the colour of the seaweed (Jiao et al., 2011). Red seaweeds are a rich source of galactan-based polysaccharides such as carrageenans and agarans. Ulvaran is the primary type of sulphated-polysaccharide found in green seaweed. The main sulphated-polysaccharides found in brown seaweeds are the fucose-containing fucans such as fucoidan and ascophyllan. Brown seaweed is also a rich source of non-sulphated polysaccharides such as laminarin and alginic acid. Fucoidan is the sulphated-polysaccharide which has been most frequently investigated for its antioxidant activity. A study found that the maximum content of fucoidan in *Ascophyllum nodosum* and *Fucus vesiculosus* were approximately 4% and 3.5% (w/w), respectively (Kim, 2012).

2.4. Antioxidant vitamins

Seaweeds are a source of both fat-soluble and water soluble vitamins. The tocopherol content of seaweed is colour-dependant. α-, β-, and γ-tocopherols are present in brown algae while green and red algae contain only α-tocopherol. Vitamin C content is not related to the colour of seaweed (Norris et al., 1937) but is influenced by extrinsic factors such as sunlight and water temperature.

2.5. Mycosporine-like amino acids (MAA)

MAA are a range of small secondary metabolites produced by seaweed and are postulated to have UV-protective and antioxidant activity among other functions. MAA are predominantly found in red and brown algae, while a limited number of green algae also produce MAA (Carreto & Carignan, 2011).

2.6. Antioxidant enzymes

Seaweeds contain a vast number of cellular antioxidant enzyme and protein systems including ascorbate peroxidase, catalase (CAT), dehydroascorbate reductase, glutathione peroxidase (GPx) and superoxide dismutase (SOD) as detailed by Collén et al. (2007).

3. Factors affecting the antioxidant content of seaweed

Due to the unpredictable nature of the marine environment, seaweed is subjected to a number of abiotic stresses. Seaweeds have developed a wide range of mechanisms to protect against these external stresses. A study by Collén et al. (2007) identified a number of antioxidant proteins and enzymes upregulated in *Chondrus crispus* in response to abiotic stress including ascorbate peroxidase, catalase (CAT), dehydroascorbate reductase, glutathione peroxidase (GPx), glutaredoxin glutathione reductase, superoxide dismutase (SOD), thioredoxin, cytochrome P450s, glutathione S-transferases, xenobiotic reductase and many other proteins. The concentration of the antioxidants in algae are dependent on a range of environmental factors such as salinity, water and air temperature, UV-light, nutrient availability, maturity and exposure to heavy metals. Genetics may also play a role in the phlorotannin content of seaweed (Jormalainen et al., 2003; Jormalainen & Honkanen, 2004).

3.1. Salinity

The salinity/salt content, measured as salt in parts per thousand (ppt), of different waters is as follows; fresh water contains < 0.5 salt ppt; brackish water contains 0.5 to 30 salt ppt and seawater contains approximately 35 salt ppt. Carotenoid, polyphenol and ascorbic acid levels and CAT and peroxidise (POX) activity in a range of algae have shown a positive correlation with increased salinity in pre (22.5 salt ppt) and post- monsoon (12.2 salt ppt) estuarine waters (Chakraborty et al., 2010). Carotenoid and chlorophyll a levels in *Dunaliella tertiolecta* also increased with increasing salinity (Fazeli et al., 2006) and the observed increases were in direct response to saline-induced stress. Other studies have also demonstrated a similar positive correlation between environmental salinity levels and polyphenol content in algae (Connan & Stengel, 2011).

3.2. UV exposure

A positive correlation between the phenol content of seaweed and their exposure to UV-light intensity has been found in a number of studies. Solar radiation is vital for photosynthesis however the presence of high energy short wavelength (UV) light can cause damage to DNA and other organelles (Bischof et al., 2006). This becomes a particular issue in the summer months when solar radiation is highest. Seaweeds have a highly developed protective mechanism against UV-damage which incorporates cellular antioxidant systems and antioxidant compounds including polyphenols and ascorbic acid. Flodin et al. (1999) found that bromophenol levels and bromoperoxidase activity were highest in *Ulva lactuca* during the summer months in Australia. In contrast, Chung et al. (2003) observed that bromophenol levels in *Padina arborescens*, *Sargassum siliquastrum*, and *Lobophora variegata* were significantly higher in winter months in the northern hemisphere. Kim (2012) found that the level and purity of fucoidan increased in *Ascophyllum nodosum* during the summer months especially in July. Further studies observed similar increases in polyphenol and phlorotannin concentrations in response to increased UV exposure during the summer months in a range of seaweeds (Abdala-Díaz et al., 2006; Pavia & Brock, 2000; Kamiya et al., 2010). Studies have found that polyphenol levels were highest during the autumn months (Kim, 2012). Honya et al. (1994) found that tocopherol and β-carotene levels in *Laminaria japonica* were greatly reduced during the winter months and maximal during the summer months in Japan.

Pinto et al. (2011) found that the levels of several carotenoids and chlorophyll a increased in *Gracilaria tenuistipitata* following exposure to high intensity light (1000 µmol photons/m^2/s^1) under laboratory conditions. In contrast, Sampath-Wiley et al. (2008) found that exposure to sunlight decreased the chlorophyll content in *Porphyra umbilicalis* inhabiting the uppermost intertidal zone. UV exposure has also been observed to increase mycosporine-like amino acids (MAAs) in seaweed. MAA are secondary metabolites, produced in algae, and are thought to have UV-absorbing, antioxidant, and osmotic balancing activities (Arrigo & Thomas, 2004). UV exposure was found to enhance MAA in *Palmaria palmata* and *Devaleraea ramentacea* (Aguilera et al., 2002; Karsten et al., 1999) and SOD activity in *Monostroma aff. arcticum* (Aguilera et al., 2002). Cronin & Hay (1996) noted that increasing light intensities decreased terpene concentrations in *Dictyota ciliolate*.

3.3. Temperature

Air and water temperatures can also alter the antioxidant profiles of algae. During cold and freezing conditions the mechanics of the Calvin cycle, the light-independent stage of photosynthesis which uses energy to convert water and carbon dioxide into organic compounds, can be severely reduced preventing the dissipation of excess energy and allowing energy to react with oxygen leading to the production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) which can cause serious cell damage (Yordanova & Popova, 2007; Demmig-Adams et al., 1996). Lohrmann et al. (2004) found that the activities of the antioxidant enzymes SOD, ascorbate peroxidise (APX), glutathione reductase (GR) and levels of vitamin C increased in *Mastocarpus stellatus* and *Chondrus crispus* during the winter months in Maine especially at temperatures below 7.5°C. Işık et al. (2006) observed similar increases in the vitamin C levels in cultured *Spirulina platensis* (a cyanobacteria) during winter months when the temperature averaged 18.6ºC, while the vitamin E content was higher during the summer months when temperatures averaged 33.9ºC. Increases in vitamin C and antioxidant enzymes may allow the algae to tolerate freezing conditions. Dhargalkar (2004) found that chlorophyll a levels in *Palmaria decipiens*, *Phyllophora antarctica*, *Porphyra endiviifolium* and *Iridaea cordata* harvested from the Antarctic increased when the cultured algae were transferred from a lower temperature range of -4°C to 0°C to higher temperature ranges of 0°C to 20°C although the viability of most of these seaweeds decreased at higher temperatures.

3.4. Desiccation

Desiccation, or dehydration, is another important factor affecting the antioxidant status of seaweed. Seaweeds that inhabit the intertidal zone are regularly subjected to emersion during low tide and are exposed to high levels of desiccation, especially during times of high levels of sunlight or drought, which can cause water losses of more than 90% leading to the production of ROS (Contreras-Porcia et al., 2010). Contreras-Porcia et al. (2010) found that desiccation induced stress increased the activity of a number of antioxidant enzymes (APX, dehydroascorbate reductase, GR, and peroxiredoxin) in *Porphyra columbina*. Sampath-Wiley et al. (2008) postulated that the elevated catalase and glutathione reductase activities and carotenoid and chlorophyll content of *Porphyra umbilicalis* upon emersion protect the seaweed from

oxidative stress allowing it to colonise the harsh environments of the intertidal zone that other species cannot tolerate. Similar elevations in carotenoids and enzymes were seen in desiccated *Gracilaria corticata* (Kumar et al., 2011). Burritt et al. (2002) found that the glutathione and ascorbic acid levels in *Stictosiphonia arbsucula* from the upper seashore recovered more successfully from desiccation-induced depletion than members of same species that normally inhabited the lower seashore which is another adaptive trait allowing seaweed to survive in harsh environments.

3.5. Heavy metals

Exposure to heavy metals has been shown to cause alterations in the antioxidant status of algae. Ryan et al. (2010) found that the polyphenolic content of *Polysiphonia lanosa* increased following cadmium-induced stress. However, other metals have been found to deplete the polyphenol content of seaweed. Connan & Stengel (2011) found that copper exposure increased the release of polyphenols to the surrounding water in *Ascophyllum nodosum* and *Fucus vesiculosus* resulting in decreased polyphenol levels in the algae. Ratkevicius et al. (2003) found that exposure to copper depleted polyphenol, glutathione and vitamin C levels in *Enteromorpha compressa* while dehydroascorbic acid (DHA) levels were enhanced indicating potent oxidative stress. Similar trends were observed by Murugan & Harish (2007) whereby exposure to heavy metal-based pollutants depleted phenol, ascorbic acid and GSH levels while increasing DHA levels and the enzyme activity of SOD and APX. Pinto et al. (2011) found that exposure to cadmium and copper elevated the levels of a range of pigments including β-carotene and chlorophyll a in *Gracilaria tenuistipitata*.

3.6. Nutrient availability

A number of studies indicate that the antioxidant status of algae may be influenced by nutrient availability and the carbon/nutrient balance hypothesis has an important role. In this hypothesis seaweed will use carbon for growth purposes when nutrients, such as nitrogen are abundant but use it to produce carbon-based compounds, like phlorotannins, when nutrient levels are limited (Van Alstyne & Pelletreau, 2000). Data reported by Pavia & Brock (2000) and Arnold et al. (1995) support this hypothesis as they observed a negative relationship between tissue nitrogen content

and phlorotannin concentrations in *Fucus vesiculosus* and *Lobophora variegata* when nitrogen levels were low, total phenol content was high but when nitrogen levels were high, TPC was low. Van Alstyne & Pelletreau (2000) found that the phlorotannin content in *Fucus gardneri* embryos decreased drastically upon enrichment with phosphorus, nitrogen and iron, although other studies have found the contrary (Hemmi et al., 2004). Cronin & Hay (1996) noted that terpene concentrations in *Dictyota ciliolata* increased with increasing nutrient availability.

3.7. Maturity

Variations in phlorotannin levels have also been reported in different tissues of the same plant (Connan et al., 2006). Pavia et al. (2003) reported that the adult tissue of *Ascophyllum nodosum* contained higher phlorotannin content than the juvenile tissue of the same plant possibly due to the juvenile investing resources into growth rather than producing compounds like phlorotannins. Pansch et al. (2008) found that the phlorotannin content was higher in the reproductive tissues of *Lessonia nigrescens* and *Macrocystis integrifolia* than in the vegetative tissues. Koivikko (2008) found that concentrations of phlorotannin were significantly higher in the physodes (subcellular organalle) than in the cell wall tissue of *Fucus vesiculosus*. Fucoidan levels were found to increase greatly in *Undaria pinnatifida* and *Costaria costata* upon maturity (Skriptsova et al., 2010; Imbs et al., 2009).

3.8 Commercial applications

The observed changes in the antioxidant profiles of seaweed caused by UV-radiation, temperature, salinity, nutrient availability, desiccation and other factors could potentially have commercial applications. To exploit the salinity and UV light-induced increases in carotenoid and polyphenol concentrations, seaweeds could be harvested in times of high water salinity, for example in pre-monsoon periods, or in times of high UV exposure, such as in the summer months. Similarly, some species of seaweed (or tissues from them) should be harvested at maturity when levels of fucoidan and phlorotannins are at their highest. Alternatively, seaweed could be cultured under controlled growth conditions using a bioreactor or similar apparatus whereby conditions such as water temperature, salinity, UV-light, nutrient availability and desiccation could be controlled to produce seaweed rich in a desired compound. Similar work has been performed using microalgae whereby

manipulation in the growing conditions of *Dunaliella salina* and *Haematococcus pluvialis* has resulted in higher yields of β-carotene and astaxanthin, respectively (García-González et al., 2005; Lorenz & Cysewski, 2003). Some studies on the use of bioreactors to grow macroalgae (Muñoz et al., 2006) have been reported but their use to manipulate the antioxidant profiles of algae have not been investigated to date. The use of heavy metals to stimulate the production of antioxidants in seaweed may not be a viable option due to the toxic nature of heavy metals which could result in a pollution and contamination risk for the resulting products.

4. Techniques for the extraction of antioxidant compounds from seaweed

The majority of antioxidants are tightly bound within the seaweed matrix; for example fucoidan and a significant amount of phlorotannins are tighly bound within the cell wall, while carotenoids are membrane-bound in the chloroplasts and chromoplasts. Therefore, these components must be extracted before their levels can be properly quantified and before they can be utilized as food ingredients or for other applications. A wide range of both simple and advanced techniques for the extraction of antioxidant compounds from algae have been developed.

The most commonly utilised type of extraction involves the use of solvent. The exact solvent extraction technique employed depends on the solubility of the desired compound, for example polar compounds like phlorotannins solubilise very easily in highly polar solvents such as water, acetone and alcohols. In contrast, non-polar or lipophilic compounds such as vitamin E and carotenoids are insoluble in water, and can only be extracted using low or non-polar solvents like hexane and chloroform. Another factor governing the extraction technique depends on the desired purity of the compound. Simple solvent extraction techniques such as maceration, infusion and more advanced methods such as Soxhlet extraction, counter-current extraction, microwave-assisted extraction (MAE), ultrasound/sonication extraction (UAE), hydrodistillation, supercritical fluid extraction (SFE), and accelerated solvent extraction (ASE) can be used to extract compounds from plants (Handa et al., 2008; Tandon & Rane, 2008). Of these techniques maceration (Lopez et al., 2010); Soxhlet extraction (Duan et al., 2006) and percolation (Ananthi et al., 2011) have been most commonly used to extract antioxidant compounds from algae. Recent studies by Rajauria et al. (2012) and Ravikumar et al. (2011) have continued to use simple solvent extractions to obtain

phenolic rich extracts from a range of seaweeds. Lim et al. (2002) and Badrinathan et al. (2011) used the Soxhlet in conjunction with a fractionation step to extract purified phenolic extracts from *Sargassum siliquastrum* and *Sargassum myriocystum*. Although these methods remain popular to date as they are relatively simple and inexpensive, they have a number of limitations such as long extraction times, large solvent volume requirement, possible thermal damage to the extracted material and potential toxicity associated with some solvents such as methanol and hexane (Wang & Weller, 2006). Modern techniques such as SFE, pressurized liquid extraction (PLE) or accelerated solvent extraction (ASE®), water extraction and particle formation on-line (WEPO®) and subcritical water extraction (SWE) are being developed to overcome the limitations associated with more established techniques (Mendiola et al., 2008).

4.1. Accelerated solvent extraction®/Pressurized liquid extraction (ASE®/ PLE)

ASE®/ PLE utilises elevated pressures (10 to 15 MPa) and temperatures (50 to 200°C) in combination with relatively low volumes of solvents to extract compounds in a short time (minutes as opposed to hours) when compared to simple enzymatic (Heo et al., 2005 a) and solvent (Cox et al., 2010) extraction methods. However, ASE®/ PLE has shown a comparable extraction yield to that of traditional extraction methods (Denery et al., 2004). ASE®/ PLE has exhibited high extraction yields of carotenoids from *Haematococcus pluvialis* at approximately 37% of the total weight of the plant (Jaime et al., 2010). The main disadvantage of this method is that thermolabile compounds may be damaged due to increased temperatures (Wang & Weller, 2006).

4.2. Solvent free extraction (SFE)

SFE allows for the extraction of bioactives using only supercritical carbon dioxide as the extraction vehicle resulting in solvent-free extracts. This method allows for the extraction of thermolabile compounds as the procedure can be performed at temperatures as low as 30°C. The main limitations associated with this method include the non-polarity of CO_2 and the expense of the equipment required and CO_2 (Ajila et al., 2011). SFE has been used on a small-scale to extract β-carotene from *Dunaliella salina* (Mendes et al., 2003). SFE has been used in the extraction of fucoxanthin and polyphenols from *Undaria pinnatifida* (Roh et al., 2008), and

chlorophyll from *Scenedesmus obliquus* (Choi et al., 1987), using ethanol as a co-solvent to aid the extraction process. Co-solvents must be used in the SFE method in order to extract polar compounds, such as polyphenols, from seaweed.

4.3. Ultrasonic-assisted extraction (UAE)

Ultrasonic-assisted extraction (UAE) is an extraction method that utilizes sonic energy to disintegrate the plant structure to release bioactive compounds in a short period of time. This method can be used to extract thermolabile compounds. UAE has been used to extract phycobiliprotein from *Porphyra yezoensis* (1.2% yield from total weight of plant) (Zhu et al., 2008) and MAA from *Porphyra* species (Tartarotti & Sommaruga, 2002) and *Palmaria palmata* (Yuan et al., 2009). Both ASE and SFE can be used in conjunction with ultrasonic-assisted extraction (UAE) to the improve extraction yield.

4.4. Microwave assisted extraction (MAE)

Microwave assisted extraction (MAE) is a technique that uses microwave energy to heat the temperature of a solvent well above its boiling point, increasing extraction efficiency and shortening the extraction time (Ballard et al., 2009). MAE has been found to be an effective method in the extraction of carotenoids from algae species which contain frustules or thick exopolysaccharide envelopes which normally limit the extraction procedure (Pasquet et al., 2011). MAE has been used to extract fucoidan from *Fucus vesiculosus* (Rodriguez-Jasso et al., 2011).

4.5 Enzymatic extraction

Enzymatic extraction methods have been used as an alternative to solvent extraction. Enzymatic extraction methods are normally used for extracting antioxidant compounds from fish derived sources such as hoki (*Johnius belengerii*) (Kim et al., 2007) and eel (*Conger myriaster*) (Ranathunga et al., 2006) but a number of proteases and carbohydrases have been used to degrade seaweed to facilitate the extraction of antioxidant compounds. Enzymatic extraction techniques are considered to be a superior extraction method both environmentally and financially, as the need for expensive and possibly toxic solvents is eliminated (Heo et al., 2003). However, a number of enzymes are identified as occupational hazards due to their potential respiratory and skin sensitising activities (Green & Beezhold, 2011).

Enzymatic extraction has been found to deliver a much higher extract yield than solvent based methods. Maximum antioxidant yields from solvent based extraction methods range from 6.5% (w/w) (Lee et al., 2010) when using simple methods, to approximately 37% (w/w) when more advanced methods like ASE®/ PLE were utilised (Jaime et al., 2010), enzymatic extraction methods have exhibited much higher yields with crude enzyme hydrolysate extracts ranging from 40% (w/w) (Heo et al., 2003) to 77% (w/w) (Wang et al., 2010). The higher extraction yield observed using enzymes may be due to the breakdown of proteins which reduces the formation of protein-polyphenol complexes which can otherwise impair the extraction of polyphenols (Wang et al., 2010). Billakanti et al. (2012) used the enzyme alginase lyase in conjunction with ethanol and dimethyl ether to increase the extraction yield of fucoxanthin from *Undaria pinnatifida* and achieved a similar extraction yield to that obtained using chloroform (Billakanti et al., 2012). Costa et al. (2011) combined enzymatic and solvent extraction procedures to extract heterofucans (fucoidan) from *Sargassum filipendula* however, the extraction yields were not reported. The main limitation of enzyme extraction is that the composition of the compound to be extracted must be known in order to utilise the correct hydrolyzing enzyme, for example a protein based or bound antioxidant would require the use of a protease to liberate it and a carbohydrate associated compound would require a carbohydrase (Mercer & Armenta, 2011).

4.6. Purification of extracts by chromatography fractionation

A range of chromatography-based fractionation techniques have been used to isolate pure compounds from crude seaweed extracts prepared using solvent and enzyme extraction methods. Fucoxanthin has been extracted from *Sargassum siliquastrum* using a combination of solvent extraction, silica column, chromatography, Sephadex chromatography and HPLC (Heo & Jeon, 2009). Ahn et al. (2007) used two simple solvent extraction steps followed by Celite column chromatography eluted by diethyl ether. The diethyl ether fraction was further purified using Sephadex chromatography and HPLC to isolate three phlorotannins; phloroglucinol, eckol and dieckol. Other studies have used somewhat similar techniques to extract phlorotannins and other polyphenols from a range of seaweed (Kang et al., 2006). Siriwardhana et al. (2004) used a simple enzymatic (Alcalase and Ultraflo) extraction followed by an ultrafiltration step to yield four antioxidant fractions.

Seaweed based antioxidants can be extracted using a variety of procedures such as simple solvent and enzyme extraction techniques or more advanced methods such as SFE, depending on a number of factors. Food grade solvents, such as acetone or ethanol, and enzymes, such as alcalase and protomex, are required for the preparation of antioxidant extracts that are intended for use as ingredients in food products. The more advanced method of SFE is an ideal extraction method for extracts intended for food grade purposes however its high costs, relatively small scale usage and unsuitability for the extraction of polar compounds may mean that more traditional methods may be more suitable for large scale extraction. Other factors such as cost, environmental issues, polarity of the extract of interest and the desired purity of the extract must be taken into account before an extraction method can be employed to extract antioxidants from seaweed. Continued research is necessary for the development of safe and efficient methods for the extraction of antioxidant compounds from seaweeds.

5.0. Measurement of the antioxidant potential of seaweed extracts

The antioxidant activity of seaweeds extracts have been investigated in a variety of *in-vitro* and *in-vivo* based experiments. The *in-vitro* antioxidant activity of seaweed extracts may be determined using chemical reaction assays or cell culture models. Chemical reaction assays are based on either hydrogen atom transfer (HAT) reactions or single electron transfer (ET) reactions which measure the ability of a compound to donate one electron. These assays often employ a substrate which will undergo a colour change upon reduction thereby allowing antioxidant activity to be quantified spectrophotometrically (Huang & Prior, 2005; Sun & Tanumihardjo, 2007). The total phenol content (TPC), ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and the Trolox equivalent antioxidant capacity (TEAC) assays are the most commonly used ET methods for the determination of the antioxidant potential of seaweed and seaweed extracts. HAT-based assays include the oxygen radical absorbance capacity (ORAC), β-carotene bleaching assay (BCBA), total radical trapping antioxidant parameter (TRAP) and total oxidant scavenging capacity (TOSC). No single assay may be regarded as the benchmark for the assessment of antioxidant capacity as each assay has certain limitations therefore it is recommended that several antioxidant assays are conducted in order to establish the overall antioxidant capacity of a sample. Studies

investigating the antioxidant capacity of seaweeds, extracted by various methods, generally employ at least three methods for the measurement of antioxidant potential. The use of chemical reaction assays to determine the antioxidant activity of brown seaweeds has been extensively reviewed in Balboa et al. (2013) and this study suggested that chemical reaction based assay are excellent preliminary tools however, more advanced methods must be used to fully understand and determine the antioxidant effects of seaweed extracts.

5.1 Total phenol content (TPC)

The majority of the antioxidant capacity of polar solvent derived seaweed extracts may be attributed to their polyphenol content therefore the TPC assay is the primary method employed for the assessment of antioxidant activity of seaweeds. The mechanism behind the TPC assay involves the reduction of Mo^{6+} (yellow) to Mo^{5+} (blue) by an antioxidant compound. The TPC assay is subject to interference from sulfites, reducing sugars, and amino acids and has a reduced accuracy for lipophillic compounds therefore TPC is not an entirely accurate measurement of total phenols (Singleton et al., 1999). The reported TPC of seaweed differs significantly between studies and is dependent on the seaweed and the extraction method employed. Recent studies have found the TPC of seaweed to range from levels of 1.2 mg GAE/g in a methanol (100%) extract prepared from *Stypocaulon scoparium* (Lopez et al., 2011) to 286 mg GAE/g in methanol (60%) extract prepared from *Himanthalia elongata* (Rajauria et al., 2012).

5.2 Oxygen radical absorbance capacity (ORAC)

The ORAC measures the ability of antioxidants to inhibit peroxyl radical-induced oxidation. The ORAC assay is not suitable for lipophilic compounds and is also sensitive to temperature. Recent studies have reported that the ORAC of seaweeds ranged from 5 trolox (synthetic vitamin E) equivalents (TE) μmol/g in protease extracts prepared from *Porphyra columbina* (Cian et al., 2012) to 150 TE μmol/g in alcalase extracts prepared from *P. palmata* (Wang et al., 2010).

5.3 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay

The DPPH radical scavenging assay measures the ability of a substance to reduce DPPH radical (purple) into a non radical form (yellow). A limitation of the DPPH

scavenging assay is the dissimilarity of the DPPH radical from any naturally occurring radicals. DPPH scavenging activity may be expressed as either the % DPPH radical scavenged, or as a half maximal effective concentration (EC_{50}). DPPH EC_{50} values have ranged from 297.7 µg/ml in methanol (100%) extracts prepared from *Himanthalia elongata* (Rajauria et al., 2012) to 0.125 µg/ml in methanol (60%) extracts prepared from *H. elongata*.

5.4 Ferric reducing power activity (FRAP) assay

In the FRAP assay the ferric ion (Fe^{3+}) (colourless) is the probe which is reduced to a ferrous ion (Fe^{2+}) (blue) in the presence of an antioxidant. A limitation of the FRAP is that it specifically measures the ferric reducing ability of a compound and not the overall antioxidant activity of the test substance. In addition the accuracy of the FRAP assay can be limited by the presence of chelators such as EDTA in test samples. The FRAP activity of extracts can be expressed in a number of ways and comparisons between different studies can be difficult. Rajauria et al. (2012) found that an extract prepared from *H. elongata* using 60% methanol had the highest FRAP activity (11.7 mg TE/g) compared to extracts prepared with water or 20%, 40%, 80% or 100% methanol. The FRAP activity correlated with the other measures of antioxidant activity (TPC and DPPH) which were also highest for the 60% methanol extract. Kelman et al. (2012) used the FRAP assay to compare the total antioxidant activity of methanol extracts prepared from 30 species of Hawaiian algae and found that *Turbinaria ornata* had the highest activity at 10.27 µM $FeSO_4 \cdot 7H_2O$/ µg extract.

5.5 Ferrous ion chelating ability (FICA)

Polyphenols are known to chelate a number of metals however their chelation of iron has been most frequently investigated (Perron & Brumaghim, 2009). The chelation of transition metals prevents them from partaking in the Fenton-reaction thereby preventing the formation of ROS. The metal chelating ability of polyphenols is dependent on their structure. Heim et al. (2002) states that the chelating ability of flavonoids is highly correlated to the position of hydroxyl, ketone and catechol groups on the structure. In terms of antioxidant peptides the presence of the amino acid histidine is highly correlated with the metal binding of peptides (Torres-Fuentes et al., 2012; Chen et al., 1998). Wang et al. (2009) observed that aqueous extracts from a range of seaweed chelated ferrous ions more effectively than acetone extracts.

Santosa et al. (2004) found that a methanol extract from *Caulerpa sertularoides* chelated ferrous ions by 55%. Budhiyanti et al. (2011) found that aqueous and butanol fractions from *Sargassum hystrix* exhibited FICA of 51.5% and 44.75%, respectively. Chew et al. (2008) compared the FICA of methanol (50%) extracts from *Padina antillarum*, *Caulerpa racemosa* and *Kappaphycus alvarezzi* and found that the *P. antillarum* extract had superior chelating ability (70%) compared to the other extracts (15% and 7.5%). Wang et al. (2008) found that at low concentrations (below 0.76 mg/mL extract) chromatographically purified fractions from *Laminaria japonica* had higher FICA compared to the crude extract. A chloroform extract from *Sargassum siliquastrum* was found to chelate iron ions by 70% (Cho et al., 2007).

5.6 β-carotene bleaching assay (BCBA)

BCBA measures the ability of antioxidants to prevent linoleic acid free radical-induced β-carotene bleaching which is indicated by a decrease in the orange colour. According to Chew et al. (2008) the main limitation of the BCBA is that only lipophilic compounds can affect the BCB inhibition however other studies have found that polar compounds can also prevent BCB (Ismail & Hong, 2002). A BCB inhibition of greater than 80% was measured in methanol-chloroform (2:1) extracts from *Symphyocladia latiuscula* and their fractions prepared using vacuum liquid chromatography (Zhang et al., 2007). A lower BCB inhibition (40%) was observed by Souza et al. (2011) in a methanolic extract prepared from *Gracilaria birdiae*. A similar range was seen in 50% methanol extracts prepared from *P. antillarum* (45%), *C. racemosa* (35%) and *K. alvarezzi* (48%). A comparison of water and ethanol extracts from the commercially available seaweed (Nori (*Porphyra* species), Kumbu (*Laminaria* species), Wakame (*Undaria* species) and Hijiki (*Hijikia* species) found that water extracts were more effective at preventing BCB (Ismail & Hong, 2002).

5.7 Additional antioxidant assays

Additional antioxidant assays that have been used to assess the antioxidant capacity of seaweed extracts include total radical trapping antioxidant parameter (TRAP), total oxyradical scavenging capacity (TOSC) and Trolox equivalence antioxidant capacity (TEAC). A limitation of the TRAP assay is that it depends on a lag phase and not all antioxidant compounds exhibit a lag phase. The TOSC assay is difficult and expensive due to the requirement of a gas chromatographer and multiple

injections. The TEAC assay has variable reaction times depending on the compound making the comparison of different compounds difficult.

No single assay can be considered as the benchmark for the assessment of antioxidant capacity as each assay has certain limitations such as the interfering effects sulfites, reducing sugars, and amino acids have on the TPC assay. Therefore, it is recommended that a number of antioxidant assays are conducted in order to establish the overall antioxidant capacity of a sample. Studies investigating the antioxidant capacity of seaweeds, extracted by various methods, generally employ at least three antioxidant assay methods for the measurement of antioxidant potential.

6. Measurement of the antioxidant potential of seaweed and seaweed extracts in cells in culture and animal models

Chemical assays can be used to estimate the antioxidant potential of seaweeds however a greater understanding of the possible antioxidant effects *in vivo* can be determined using cells in culture or animal cell models. Tables 1 and 3 detail the overall antioxidant effects of seaweed extracts in cells in culture and animal models, respectively and tables 2 and 4 detail studies which have investigated the ability of seaweed extracts to protect against oxidant-induced DNA damage in cells in culture and in animal models, respectively.

6.1 Seaweeds and seaweed extracts investigated in cell and animal model systems

Japan is the world leader in the field of seaweed research therefore the majority of seaweeds that have been added to cells or administered to animals for an investigation of their antioxidant effects are those which are endemic to the coasts of Japan and neighbouring countries such as China and Korea (Tables 1-4). The majority of seaweeds investigated were brown or red species as brown species are known to contain the highly antioxidant compounds, phlorotannins and red seaweeds contain high concentrations of sulphated-polysaccharides.

In general, an extract of the seaweed was prepared prior to its addition to cells or use in animal feeding trials. A simple solvent extract was investigated in a number of studies. The solvents used in the preparation of antioxidant extracts from seaweeds should ideally be food grade when they are to be used in animal feeding trials. Extracts prepared using water have been added to cells in culture (Fallarero et al., 2003, Yang et al., 2012) and have also been fed to rats (Sathivel et al., 2008).

Seaweed extracts, prepared using ethanol, have also been fed to rats (Raghavendran et al., 2005; Raghavendran et al., 2007). Methanol, which is toxic and not food grade, was used to prepare extracts from seaweeds which were subsequently fed to fish (Nagarani et al., 2012) and added to human keratinocytes, HaCaT cells (Ko et al., 2011). Prior to their use in a cell model or animal system, solvent-derived extracts are generally dried to a powder which removes all but trace amounts of the solvent. These extracts are then generally added into the animal feed directly, however; a study by Schultz Moreira et al. (2011) incorporated the seaweed *Himanthalia elongata* into a reconstituted pork product for a rat feeding trial (Table 3).

Enzymes have also been used to extract antioxidants from seaweed for an investigation of the seaweeds cellular antioxidant effects. The two main classes of enzymes which have been used are proteases and carbohydrases. The advantages of preparing an enzymatic extract are an increased extraction yield. A range of carbohydrases (AMG, Celluclast, Termamyl, Ultraflo, Viscozyme and Maltogenase) and proteases (Protamex, Kojizyme, Neutrase, Flavourzyme and Alcalase) have been used in the preparation of extracts which were subsequently added to cells in culture (Table 2). The use of enzymatic antioxidant extracts in animal trials has not been reported.

Several purified compounds including phlorotannins, sulphated polysaccharides, carotenoids, mycosporine-like amino acids (MAA), monoterpene lactone (loliolide) and phycobiliprotein have been isolated from seaweed and added to cells in culture (Table 1 and 2). *Ecklonia cava* is a particularly rich source of phlorotannins and contains a diverse range of these compounds including Triphlorethol-A, 1,3,5-trihydroxybenzene, pyrogallol-phloroglucinol-6,6'-bieckol, phloroglucinol, eckol and dieckol. Diphlorethohydroxycarmalol from *Ishige okamurae* is another phlorotannin that has been tested in cells in culture (Tables 1 and 2). Fucoidan has been the compound predominantly investigated in animal feeding trials. A possible reason for this may be due to the relatively simple extraction method required for the extraction of crude fucoidan (Chotigeat et al., 2004). In addition, the yield of fucoidan from seaweed is significantly higher compared to compounds such as purified phlorotannins, 2.75% (w/w) of crude fucoidan has been extracted from seaweed (Chotigeat et al., 2004) in comparison to 0.0055% (w/w) Triphlorethol-A (Kang et al., 2006).

6.2 Antioxidant effects of seaweed extracts

The antioxidant effects of seaweed and seaweed extracts have been investigated in a broad range of cell types including human skin, fibroblast, lung and lymphocytes; rodent brain, T-cells, lung, lymphocytes and macrophages and monkey kidney cells (Table 1 and 2). Their antioxidant activities were also assessed using animal models which mostly involved the use of rat or mouse feeding trials, however in a limited number of studies the seaweed extracts were administered to the animals intravenously (Tables 3 and 4). The antioxidant effects of the seaweed extracts were then determined in various cells harvested from the animals following the desired feeding period. As is the case in many pharmaceutical and toxicological studies, the majority of studies selected hepatocytes as the main cell type for investigation which is due to the presence of major drug and xenobiotic-metabolizing enzymes such as the cytochrome P450 enzyme superfamily (Guo et al., 2011). Additional target organs investigated included brain, kidney and plasma cells.

Seaweed and seaweed extracts were found to ameliorate the increase in reactive oxygen species (ROS) generation induced by oxidants H_2O_2, UV-B and methyl mercury in human and animal cell models. Seaweed, delivered orally, intravenously or topically also increased the activity of the antioxidant enzymes including CAT, SOD, GPx in the cells of animal models and prevented the decrease in antioxidant enzyme activity induced by xenobiotics such as ethanol, tetrachloride, paracetamol, γ-radiation and cyclosporine A (Table 3). Seaweed protected against the DNA damage induced by H_2O_2, UV-B and γ-radiation in various cell types in culture (Table 2) and against the DNA damage induced by mercury, H_2O_2, cyclophosphamide and paracetamol in the cells of fish, mice and rats (Table 4).

H_2O_2 is the most commonly employed oxidant in studies investigating DNA damage because it is a physiological oxidant, naturally produced in oxidative metabolism, and its damaging mechanism, especially to DNA, is well established (Barbouti et al., 2001). Similarly the damaging effects of UV radiation which is caused either by direct DNA damage through interaction with DNA bases or indirect damage through the formation of ROS such as singlet oxygen and H_2O_2 has been established (Abdel-Malek et al., 2009). The main mechanism in the cellular protective ability of antioxidants is through the chelation of transition metals such as iron and copper. Seaweeds contain a number of metal chelating components such as

polysaccharides, proteins (Murphy et al., 2007) and polyphenols (Perron & Brumaghim, 2009).

6.3 Human studies

A limited number of studies have investigated the antioxidant effects of seaweed and seaweed extracts in human trials (Table 3). The daily consumption of 48 g of a seaweed supplement containing equal parts sea tangle (*Laminaria japonica*) and sea mustard (*Undaria pinnatifida*) resulted in enhanced cellular antioxidant enzyme activity and reduced lipid oxidation in red blood cells isolated from diabetic patients (Kim et al., 2008). Similarly, consumption of 1.5 g of fermented *Laminaria japonica* for each day over a five week period resulted in an increased antioxidant enzyme activity in the hepatocytes of patients suffering from high levels of γ-glutamyltransferse (γ-GT), a condition that is associated with a number of diseases (Kang et al., 2012). It has also been found that the topical application of MAA extracted from *Porphyra umbilicalis* reduced UV-A induced lipid oxidation in human skin cells (Schmid et al., 2006).

7. Dairy products as potential vehicles for functional ingredients

The European Commission defines a functional food as "a food that beneficially affects one or more target functions in the body beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being and/or a reduction in disease risk. Milk and dairy products are commonly consumed and widely perceived as healthy foods due to the high content of calcium, protein, riboflavin, vitamin B_6 and vitamin B_{12} (Fox & McSweeney, 1998). Milk is one of the most commonly fortified food products in the world and is generally used as a vehicle for vitamins D and A. The physical properties of milk and dairy products also make them excellent potential carriers of functional food ingredients. Supplementary fatty acids and fat soluble vitamins (D, K, E) readily solubilise in the milk fat globule and water soluble minerals and vitamins are dissolved in the continuous serum phase of milk. The low pH and fermented nature of yogurt makes it a more suitable vehicle for ingredients such as probiotics. In addition, yogurt is more suitable than fluid milk as a carrier vehicle for less soluble additives such as probiotics, fibre, prebiotics and phytosterols due to its gel structure which maintains

the even distribution of these ingredients. Several factors require consideration when a functional ingredient is to be incorporated into a dairy product.

7.1 Solubility of functional ingredients

The solubility of an ingredient in a carrier vehicle is governed by number of factors including polarity, composition, temperature and pH. The solubility of carbohydrate and protein (Pelegrine & Gasparetto, 2005) ingredients can be dependent on the pH of the carrier and composition of the ingredient. Varum et al. (1994) found that the solubility of chitosans significantly decreased between pH 6 and 7.5, but the solubility of chitosan in water could be improved through the addition of acetyl functional groups to the structure. The solubility of protein and peptide ingredients is highly dependent on the processing conditions (Schmidt et al., 1984); the amino acid composition of the ingredient (Trevino et al., 2007), and the pH and salinity of the carrier. Studies have found the solubility of polyphenols increases with increasing levels of glycosylation (Pellegrini et al., 2006). Functional ingredients may be manipulated to enhance their solubility, for example, the prebiotic inulin has exhibited low solubility at refrigeration temperatures (Phelps, 1965) however, enzymatic hydrolysation of inulin yields highly soluble oligofructose fractions (Franck, 2002). Hydroscopic carbohydrate-rich plant extracts such as those from seaweed are vulnerable to collapse, caking and stickiness which can significantly impair their solubility. Appropriate processing and storage are necessary to improve the solubility of plant extracts (Maltini et al., 2003). The most utilised technique to reduce caking and improve solubility is microencapsulation. Microencapsulation involves the incorporation of food ingredients into small, relatively inert soluble capsules (Desai et al., 2005). The most common type of microencapsulation involves spray-drying, however freeze-drying is utilised for heat-labile materials and is commonly used in the production of seaweed extracts (Amorim et al., 2012).

7.2 Effect of functional ingredient on stability of the carrier vehicle

The addition of functional food ingredients may affect the shelf-life of the carrier food. Antioxidants by their nature should extend the shelf life of food as has been observed for goat meat and pork patties enriched with 2% broccoli extract (Banerjee et al., 2012) or 0.02% herb extract (Hernández-Hernández et al., 2009). However, the addition of seaweed antioxidant extracts has been reported to induce pro-oxidant

effects in foods such as tilapia meat (Cabral et al., 2011) and pork meat (Moroney et al., 2011). The unstable nature of unsaturated fatty acids has been reported to negatively influence the shelf-life of the carrier vehicle in a number of studies. Let et al. (2004) found that the addition of neat cod-liver oil significantly increased lipid oxidation in milk; however this problem was resolved through the use of a cod-liver-oil-in-water emulsion. The incorporation of antioxidants prevented an omega-3 algal oil-induced increase in lipid oxidation in pork sausages, ham and turkey burgers (Lee et al., 2006a; Lee et al., 2006b). The development of microencapsulated ferrous sulphate and ferrous fumarate has been used to reduce iron-induced flavour and oxidation problems in food such as milk (Kwak et al., 2003) and yogurt (Kim et al., 2003). Microencapsulation has also been used to improve the stability of a number of ingredients such as vitamins and probiotics (Wilson & Shah, 2007). A contamination of plant-derived extracts with trace amounts of metals such as iron may cause oxidation risk in foods. Moroney et al. (2012) attributed the increased rate of oxidation in seaweed extract-enriched pork to the presence of trace levels of iron in the extract.

7.3 Effect of functional ingredient addition on consumer acceptability

Regardless of the health benefits attributed to a functional ingredient if it significantly impacts on the sensory properties of the carrier food it will not be accepted by consumers (Siegrist et al., 2008). Let et al. (2007) found that the addition of 1% cod liver oil imparted a negative fishy flavour in milk. A similar fishy flavour was detected in strawberry yogurts containing 1.84% algal-oil emulsion, however it did not negatively affect the overall liking of the products (Chee et al., 2005). The addition of 1.5% or 4.5% (w/w) fibre from date fruit or wheat bran negatively impacted on the sensory attributes of yogurt in terms of flavour and overall acceptability (Hashim et al., 2009). Sanz et al. (2008) found that enrichment of yogurt with fibre asparagus extracts negatively affected the flavour and colour of yogurt, alterations to yogurt colour and flavour varied depending on the extraction method used in the preparation of the extract. In a number of studies the addition of iron has had a detrimental effect on the sensory properties of foods, especially dairy foods (Abbasi & Azari, 2011). Kim et al. (2003) incorporated vitamin C into microencapsulated iron for addition to yogurts and found a significant increase in the overall acceptability in comparison to yogurt without vitamin C. The

astringent and bitter nature and colour of polyphenols have been found to negatively impact on the sensory attributes of a number of carrier foods such as UHT milk (Axten et al., 2008) and smoothies (Sun-Waterhouse et al., 2010). Traditionally flavours, sweeteners and colours were used to mask the undesirable attributes of polyphenols. Recently the technology of microencapsulation has been applied to polyphenols to mask their bitterness, astringency and colour when used as ingredients in yogurt (Petrotos et al., 2012). This technology could potentially be applied to produce microencapsulated seaweed extracts for use as functional ingredients in dairy foods and other products.

7.4 Bioavailability of functional ingredients

The physiological health benefits of a functional ingredient are dependent on its bioavailability. The bioavailability of polyphenols is dependent on their structure and ranges from 2 to 20% (Manach et al., 2005; Hu, 2007). The bioavailability of an ingredient can be significantly affected by the presence of other constituents in the carrier food. The bioavailability of calcium is improved in the presence of vitamin D (Mortensen & Charles, 1996). The bioavailability of iron is significantly improved in the presence of substances such as vitamin C and alcohol and decreased in the presence of polyphenols, phytate, calcium, milk proteins and fibre (Hunt, 2003). The formation of polyphenol-iron and polyphenol-protein complexes in food (Gomez-Guillen et al., 2007) has been reported to reduce the bioavailability of both polyphenols and iron (Hallberg & Hulthén, 2000; Alexandropoulou et al., 2006). Microencapsulation of iron and/or polyphenols may be a possible mechanism for enhancing their bioavailability (Augustin & Sanguansri, 2008) however Liyanage & Zlotkin (2002) found that encapsulation reduced the bioavailability of iron in cereal-based foods. To maximise the bioavailability, functional ingredients should either be incorporated into carrier foods which are free of any interacting constituents or alternatively ingredients can be microencapsulated to minimise interaction with other components of the food.

7.5 Possible toxicity risk from functional ingredient addition

Although the majority of commonly consumed foods such as fruit and vegetables are considered GRAS (generally recognised as safe; FDA), concentrated extracts from these sources may not be safe due to the presence of elevated concentrations of

particular compounds (Milner, 1999) and it is necessary to determine the levels at which extracts can be safely added to foods. Most seaweed when consumed in small amounts cause little or no health problems however highly concentrated seaweed powders, such as those used in Chinese medicine, can contain iodine levels far exceeding the safe upper limits set by the Dietary Reference Index (Otten et al., 2006). The consumption of seaweed extracts has been documented to cause hyperthyroidism (overactive thyroid) (Shilo & Hirsch, 1986) and specialised processing techniques can be used to significantly reduce the iodine content of seaweed (Teas et al., 2004). Prior to the additional of functional extracts to a food product, the extracts should be tested to determine suitable concentrations which will not cause ill health. Chromatography, mass spectrometry or similar technology can be used to accurately determine the exact composition of an extract and the safe levels of the various constituents can be determined from published studies (Kruger & Mann, 2003). Cell culture models can be employed to assess the possible toxicity of ingredients at a cellular level and have been previously used to measure the toxicity of plant-derived functional ingredients (Glei et al., 2003; Ugartondo et al., 2006). A more complete evaluation of the toxic effects of ingredients at physiological conditions may be determined through the use of animal trials which can detect potential organ specific toxicity. However, toxicology results from cell-based models and animal trials cannot be accurately extrapolated to humans when evaluating the safety of functional ingredients and therefore the use of human clinical trials may be required (Kruger & Mann, 2003).

Plants readily take up and accumulate many different heavy metals from the environment (Peralta-Videa et al., 2009) which may pose a risk especially in concentrated extracts. Studies have found elevated levels of heavy metals in terrestrial plants (Peralta-Videa et al., 2009) and seaweed (Almela et al., 2002; Wei et al., 2003). Other potential contaminants such as radioactive nucleotides (Oliver et al., 2006, Ilus et al., 1999) and microalgae-produced toxins (Marshall & Vogt, 1998) have been identified in seaweed. Therefore, potential ingredients must be tested for the presence of heavy metals, toxins and other contaminants prior to their use in foods. The presence of heavy metals in plants can be determined using atomic absorption spectrophotometry (Lokeshwari & Chandrappa, 2006), while the presence of toxins can be determined using chromatography, ELISA and other techniques (Turner et al., 2009).

The demand for functional food products continues to grow and this may be attributed to the increasing cost of healthcare, the steady increase in life expectancy, and increased understanding of the link between nutrition and health (Siro et al., 2008). As a result a number of materials have been investigated for their potential use as functional ingredients. For incorporation into liquid carrier foods such as milk, functional ingredients must be soluble at the storage temperature of the product and must not cause precipitation problems. Non-soluble components such as seaweed fiber must remain evenly distributed in dairy products like yogurt and must not sediment. An effective ingredient must remain stable and bioavailable throughout storage and must not affect the stability of the carrier food. The sensory attributes of the carrier food must not be negatively affected by the addition of functional ingredients. Prior to incorporation into a functional food, cellular and analytical chemistry methods should be undertaken to determine the potential toxicity and possible contamination of the ingredients.

8. Other issues associated with the seaweed industry

8.1 Sustainability

The sustainability of the seaweed industry is sensitive to a number of factors. Similar to any other natural resource seaweed is susceptible to over harvesting; various seaweeds in the Caribbean have declined in number due to overuse (Smith & Renard, 2002). According to Guiry (www.seaweed.ie) the sustainability of aquaculture depends on the seaweed species. Climate change such as global warming is postulated to have a detrimental effect on seaweed. A study by Campbell et al. (2011) found that warmer waters were positively correlated with higher frequencies of a microbial bleaching disease in *Delisea pulchra*. Pollution from nutrient rich effluent can cause the overgrowth or bloom of algae such as dinoflagellate leading to oxygen depletion of the water column which can kill native seaweeds (Dundas et al., 1989). Non-native or invasive seaweed species are easily introduced into new environments deliberately or accidentally from aquarium discharges, aquaculture activities and from shipping ballast water. Schaffelke & Hewitt (2007) reviewed the possible environmental effects of invasive seaweed and found that many invasive species outcompeted and displaced native species and could eliminate native species.

8.2 Legislation

Not all seaweed species have been authorized as fit for human consumption and authorization can differ from country to country. In 1999, France was the first EU country to establish a specific regulation concerning the use of seaweeds for human consumption and authorized 12 macroalgae as fit for human consumption. However in Canada and USA the majority of seaweeds are deemed suitable for human consumption. Any health claim associated with seaweed or any other product is stringently regulated by relevant food authorities, the FDA in the USA and the EFSA in the EU. Companies must apply to the relevant authority for permission before any health claim can be made in the labeling and marketing of functional food products.

Objective

Seaweed extracts and pure compounds derived from seaweed have demonstrated antioxidant activity at a cellular level by scavenging ROS, enhancing the cell antioxidant defence system and protecting DNA from oxidant-induced damage which indicates that the inclusion of seaweed in the diet may be of benefit to health. Therefore, the objective of this thesis was to investigate the potential *in-vitro* and cellular antioxidant activity of a range of extracts prepared from Irish seaweeds. The extracts demonstrating the greatest antioxidant potential were selected for addition to yogurt and milk products to investigate the feasibility of using seaweed as an ingredient for neutraceutical products.

Chapter 2 of this thesis examined the antioxidant activity of 60% methanol extracts from *Ascophyllum nodosum, Laminaria hyperborea, Pelvetia canaliculata, Fucus vesiculosus* and *Fucus serratus* sourced from west coast of Ireland. The antioxidant activity of the extracts was established using a range of chemical reaction based antioxidant assays such as the Ferric reducing antioxidant power (FRAP), β-carotene bleaching assay (BCBA), total phenol content TPC and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay. The DNA-protective effects of the extracts against H_2O_2-induced damage in Caco-2 cells were assessed using the Alkaline Single Cell Gel Electrophoresis (Comet) assay. The effect of the seaweed extracts on the SOD and CAT activity and GSH content in the human adenocarcinoma cell line, Caco-2, was assessed.

Chapter 3 of the thesis examined the protective effects of the extracts against H_2O_2 and *tert*-BOOH-induced DNA damage in Caco-2 cells and compared the protective effects against those of several metal chelators.

Chapter 4 investigated the antioxidant activities of *Ascophyllum nodosum*, *Fucus vesiculosus* and *Fucus serratus* extracted using 100% H_2O, 60% ethanol: 40% H_2O, 80% ethanol: 20% H_2O or 60% methanol: 40% H_2O in combination with an accelerated solvent extraction (ASE®) technique. The antioxidant activity of the resulting extracts was assessed using the TPC and ferrous-ion chelating ability (FICA) assay. The effects of the extracts against H_2O_2 and *tert*-BOOH-induced DNA damage in Caco-2 cells were assessed. The effect of the seaweed extracts on the SOD and CAT activity and GSH content in Caco-2 cells was assessed.

Chapter 5 investigated the feasibility of producing seaweed extract-enriched milk. The microbial status, DPPH activity, physicochemical and sensory attributes were measured over 12 days. The FICA of the milk fractions was assessed. The milk was subjected to an *in-vitro* digestion procedure and the protective effect of the milk and digestates against H_2O_2-induced DNA damage was assessed using the Comet assay.

Chapter 6 of the thesis investigated the feasibility of producing seaweed extract-enriched yogurts. The microbial status, DPPH activity, physicochemical and sensory attributes were measured over 28 days. The FICA of the yogurt fractions was assessed. The yogurts were subjected to an *in-vitro* digestion procedure. The protective effects of the yogurt and yogurt digestates against H_2O_2-induced DNA damage was assessed using the Comet assay.

In an additional study (Chapter 7), the techniques developed for this thesis were employed to assess the antioxidant activity from a range of commercially available algae-based supplements.

This PhD was funded by The Marine Functional Foods Research Initiative (NutraMara) programme. NutraMara is a programme for marine based functional food development established by the Marine Institute and the Department of Agriculture, Food and Marine. NutraMara is focused on the development of new products and ingredients from Irish based fish processing waste, seaweeds and microalgae and its objectives include the creation of a strong, interdisciplinary research capability to exploit marine biodiversity as a source of materials for use in functional foods. The NutraMara project has focused on the extraction, and biological and chemical characterisation of polyphenols, peptides, polysaccharides,

amino acids, polyunsaturated fatty acids, protein hydrolysates and materials with antioxidant, probiotic or prebiotic properties. Small scale, pilot intervention studies have been conducted on model meat and dairy products enhanced with marine derived bioactive ingredients (Chee et al., 2005; Moroney et al., 2012). The NutraMara project consists of a number of research centres, the programme is led by Teagasc Food Research Centre Ashtown (TFSCA) and works in collaboration with six other research members including: University College Cork (UCC), University College Dublin (UCD), The National University of Ireland, Galway (NUI Galway), University of Ulster (UU), University of Limerick (UL) and Teagasc Food Research Centre, Moorepark, Cork (TFRCMC). The data presented in the present thesis was obtained from samples identified and harvested by NUI Galway, which were forwarded to TFSCA for extraction. The seaweed extracts were then sent to UCC where the analysis reported in the present thesis was conducted.

According to the Sea Change Strategy (2006), the Irish seaweed production and processing sector will be worth approximately €30 million per annum by 2020, and collaborations such as NutraMara will facilitate these projections. This thesis reports the antioxidant capacity of the seaweed extracts provided by TFSCA and the influence of extraction technique on the antioxidant capacity. The overall aim of the project was to identify new functional ingredients to improve the shelf-life, quality and health properties of food products.

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CHAPTER 2

Antioxidant activities assessed using *in-vitro* and cellular model systems of five brown seaweeds harvested in spring from the west coast of Ireland.

Based on:

O'Sullivan, A.M., O'Callaghan, Y.C., O'Grady, M.N., Queguineur, B., Hanniffy, D., Troy, D.J., Kerry, J.P. & O'Brien, N.M. (2011). *In-vitro* and cellular antioxidant activities of seaweed extracts prepared from five brown seaweeds harvested in spring from the west coast of Ireland. *Food Chemistry, 126*, 1064-1070.

Abstract

Methanolic extracts of five species of brown seaweed *Ascophyllum nodosum, Laminaria hyperborea, Pelvetia canaliculata, Fucus vesiculosus* and *Fucus serratus* sourced from the west coast of Ireland were prepared. Antioxidant potential of the five seaweeds was assessed using the Total Phenol Content assay, Ferric Reducing Antioxidant Power (FRAP) assay, β-carotene bleaching assay and the DPPH• (2,2-diphenyl-2-picrylhydrazyl hydrate) scavenging assay. *A. nodosum, P canaliculata, F serratus* contained the highest phenol concentrations while *F. vesiculosus* and *F. serratus* exhibited the highest FRAP activities. *F. vesiculosus* and *A. nodosum* were the most effective exacts at both scavenging DPPH radicals and protecting against β-carotene bleaching. The antioxidant activity of the seaweed extracts was also evaluated in a cellular model using Caco-2 cells. Caco-2 cells were supplemented with increasing concentrations of the seaweed extracts for 24 hrs and cell viability was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. None of the extracts were toxic to the cells at concentrations below 2 mg/mL. The effects of the extracts on the antioxidant status of the cells were assessed by measuring reduced glutathione (GSH) content following 24 hrs of exposure. All extracts significantly ($P < 0.05$) increased GSH content in the cells. To examine cytoprotective and genoprotective effects of seaweed extracts, Caco-2 cells were pre-treated with seaweed extract for 24 hrs followed by exposure to hydrogen peroxide (H_2O_2). The antioxidant enzyme activity (catalase and superoxide dismutase) was assessed and DNA damage was measured using the comet assay. *P. canaliculata* was the most effective extract at preventing H_2O_2-mediated SOD depletion in Caco-2 cells while *F. serratus* exhibited the best DNA protective and GSH enhancing effects.

1. Introduction

Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), hydroxyl radical (OH•), superoxide anion (O_2^-) and nitric oxide (NO) are deleterious to various physiologically important molecules including proteins, lipids and DNA (Wijeratne et al., 2005). ROS, generated in living organisms during metabolism, are very unstable and highly reactive, and they tend to initiate chain reactions which result in irreversible chemical changes in proteins or lipids. These deleterious reactions can result in cellular dysfunction and cytotoxicity. A number of cellular defence systems have evolved to counteract the accumulation of ROS. These include enzymatic scavengers such as catalase, glutathione peroxide, and superoxide dismutase (SOD). Antioxidant enzymes are the primary defence system directly involved in the detoxification of ROS (Aruoma et al., 1998). Physiological and dietary antioxidants constitute non-enzymatic antioxidant defence systems, which are the secondary defence system and include glutathione, carotenoids, polyphenols and other non-nutrient compounds (Aruoma et al., 1998).

Oxidative stress is implicated in a wide variety of diseases, including the development of various human chronic diseases such as CVD, certain cancers, and a number of neurodegenerative diseases (Aruoma et al., 1998). Therefore, over the last two decades, a wide range of phytochemicals from terrestrial plant materials have been evaluated for their ability to protect tissues against oxygen-induced damage and hence lower the risk of human chronic diseases (Halvorsen et al., 2006). Oxidative stress not only affects the body but can also have detrimental effects on foods due to the production of rancid flavours and odours while also reducing the shelf-life, nutritional quality, and safety of food products (Zainol et al., 2003; Chanwitheesuk et al., 2005). In order to lower the risk of oxidative deterioration, synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been added to many food products. Due to safety issues and consumer demand, there has been considerable interest in replacing synthetic antioxidants with natural plant-based alternatives (Devi et al., 2008). Natural antioxidants derived from marine sources may have potential for inclusion in fish and meat products as alternatives to synthetic antioxidants such as BHA and BHT. A number of studies have found the antioxidant activities of seaweed extracts are greater than or on-par with that of BHT or BHA (Zubia et al., 2007; Devi et al., 2008) suggesting their potential use as replacements to artificial antioxidants.

Many types of seaweed contain a wide range of bioactive compounds with potential antioxidant activity, for example phlorotannins a range of polyphenolic compounds exclusively found in brown seaweed, can account for between 1% and 10% of the dry weight of these seaweeds (Ragan & Glombitza, 1986). Polyphenols derived from seaweeds may be more potent than analogous polyphenols derived from terrestrial plant sources due to presence of up to eight interconnected phenol rings (Hemat, 2007). In addition, compounds identified in seaweeds including alkaloids, terpenes, ascorbic acid, tocopherols and carotenoids have demonstrated antioxidant activity in a variety of *in-vitro* studies (Heo et al., 2009; Hu et al., 2008).

In contrast to terrestrial plant materials, less research has been conducted on the antioxidant potential of marine seaweeds. In recent years a number of studies reported that seaweed extracts demonstrate strong antioxidant properties (Gamal-Eldeen et al., 2009). A rich diversity of seaweed exists around the coast of Ireland with over 500 different species documented. Wild stocks of seaweed in Ireland yield approximately 36,000 tonnes annually while world output stands at close to 8 million tonnes (Werner et al., 2004; McHugh, 2003). The extent of the Irish seaweed resource has not been fully determined. Furthermore, reports on the potential antioxidant properties of seaweeds extracts from the coast of Ireland are limited.

In this study, five species of seaweed harvested from the West coast of Ireland in spring were examined for their antioxidant potential. These included *Ascophyllum nodosum*; a mid shore species found on sheltered coastlines, *Laminaria*. *hyperborea*; a lower shore species, *Pelvetia canaliculata*; an upper shore species, and two *Fucus* species *Fucus vesiculosus*; mid shore and *Fucus serratus*; a lower shore species.

The objectives of the present study were to: first, measure the antioxidant capacity of the five different seaweed extracts using a range of chemical reaction based assays. Second, investigate the cytotoxicity and antioxidant potential of the extracts using a human colon carcinoma cell line, Caco-2 cells, as a model system. In the present study, the five extracts were investigated for their potential cytoprotective and genoprotective effects against H_2O_2-induced stress in Caco-2 cells.

2. Methods

2.1. Materials

Human colon adenocarcinoma Caco-2 cells were purchased from European Collection of Animal Cell cultures (Salisbury, UK). Foetal bovine serum was purchased from Invitrogen (Paisley, Scotland). Cell culture plastics were purchased from Cruinn Diagnostics (Greiner Bio-One, Frickenhausen, Germany). Seaweed extracts were prepared in the National Seaweed Centre in NUI Galway. Calbiochem Catalase Colorimetric Activity Kit and Calbiochem SOD Assay Kit II were purchased from Merck Chemicals Ltd. (Nottingham, UK). All other cell culture chemicals and reagents were purchased from Sigma Chemical Co. (Dublin, Ireland).

2.2. Preparation of seaweeds extracts

Seaweeds were harvested from the seashore in Galway in spring and washed to remove any undesired material. The seaweeds were chopped and freezed-dried for storage. The seaweed extracts were prepared according to the method of Connan et al. (2004) with modifications. Seaweed samples were ground in a mortar with liquid nitrogen, and mixed with 75 mL of 60% methanol/ 40% distilled water. This mixture was homogenised at 24000 rpm for 1 minute and was then placed in the dark at 40°C for 3 hrs, after which the mixture was centrifuged at 9000 rpm for 15 mins. The mixture was then filtered with cotton wool to extract the supernatant which was subsequently subjected to solvent evaporation to remove methanol. The extract was freeze-dried and stored in desiccators. Samples were reconstituted in 10 mL distilled water yielding final concentrations of 10.0, 12.3, 11.0, 14.6 and 13.0 (mg/mL) of *A. nodosum, L. hyperborea, P. canaliculata, F. vesiculosus* and *F. serratus,* respectively.

2.3. Determination of Total Phenol Content (TPC)

The TPC of the extracts was quantified according to the method of Singleton & Rossi (1965). Gallic acid was used as a standard and TPC was expressed in terms of mg gallic acid equivalents / g dry weight of sample (GAEq/gdw).

2.4. Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP assay was performed according to the method of Benzie & Strain (1999). Ascorbic acid was used as a standard and FRAP values were expressed as µM ascorbic acid equivalents / g dry sample (AAEq/gdw).

2.5. DPPH• (2,2-diphenyl-2-picrylhydrazyl hydrate) scavenging assay

The DPPH assay was performed according to the method of Brand-Williams (1995) and the results were expressed as % DPPH radical scavenging activity and calculated as follows:

DPPH activity, % = (1 - ((Abs_{515nm} Seaweed extract / Abs_{515nm} control)) × 100

2.6. β–carotene Bleaching Assay (BCBA)

The BCBA was performed based on the method by Duan et al. (2006) with some modifications. Briefly, oxygenated water was mixed with β–carotene, Tween-40 and linoleic acid. The mixture (200 µL) was incubated with or without 50 µl of seaweed extract for 180 mins at 50°C and the absorbance was read at 450 nm at time zero and every 30 mins thereafter. The results were expressed as % β–carotene bleaching inhibition.

2.7. Cell culture

Human colon adenocarcinoma Caco-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with FBS (10% v/v) and non-essential amino acids (1% v/v). Cells were incubated in an atmosphere of CO_2–air (5:95, v/v) at 37°C and were maintained in the absence of antibiotics. Caco-2 cells were plated at a density of 1×10^5 cells/mL, depending on the experimental procedure. After 24 hrs the growth media was replaced with fresh media containing reduced FBS (2.5% v/v) which were supplemented with 100 µg/mL seaweed extracts for 24 hrs in 6-well plates with a final volume of 4 mL (unless otherwise stated). The cells were subjected to oxidative stress using 200 µM H_2O_2 in FBS-free media for 30 min at 37°C followed by harvesting, sonication and centrifugation. The antioxidant status of the cells was subsequently determined by measuring the activity of Catalase (CAT) and Superoxide Dismutases (SOD).

2.8. Cell viability

Caco-2 cells were supplemented with increasing concentrations (0–5.5 mg/mL) of seaweed extracts for 24 hrs in 96-well plates with a final volume of 100 μL. Cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (MTT I proliferation kit, Roche Diagnostics; Burgess Hill, West Sussex, UK) as described by Phelan et al. (2010).

2.9. Catalase (CAT) activity

Catalase activity was determined using a Calbiochem catalase colorimetric activity kit (Merck Chemicals Ltd., Nottingham, UK). This kit utilises the peroxidative function of catalase for determination of enzyme activity and is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. Results were expressed as units mg^{-1} protein where protein content was determined by the BCA method (Smith et al., 1985). One unit of catalase activity is defined as the amount of enzyme required to produce 1 nM formaldehyde min^{-1} at 25 °C.

2.10. Superoxide dismutases (SOD) activity

SOD activity was determined using a Calbiochem SOD Assay Kit II (Merck Chemicals Ltd., Nottingham, UK). This kit utilises a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. Results were expressed as units mg^{-1} protein where protein content was determined by the BCA method (Smith et al., 1985). One unit of SOD is defined as the amount of enzyme needed to induce 50% dismutation of the superoxide radical.

2.11. Reduced glutathione (GSH) content

GSH was determined by the method of Hissin & Hilf (1976). Briefly, perchloric acid (15%, v/v) was added to the cell sonicates and samples were centrifuged at 14000 rpm for 30 min at 4°C. The final GSH assay mixture contained 100 μL sample, 1.8 mL sodium phosphate-ethylenediamine tetraacetic acid buffer, pH 8, and 100 μL o-phthaldialdehyde (1 mg/mL). Fluorescence was detected at 430 nm following excitation at 360 nm. GSH levels (nmol) were expressed relative to the protein content (mg), as determined by the bicinchoninic acid (BCA) method (Smith et al., 1985)

2.12. Determination of DNA damage (Comet assay)

Caco-2 cells were supplemented with 100 µg/mL seaweed extracts for 24 hrs in 6-well plates with a final volume of 2 mL. Following incubation, the cells were treated with or without 50 µM H_2O_2 for 30 min at 37°C. Cells were harvested, suspended in a low melting point (LMP) agarose and placed on microscope slides which were precoated with normal gelling agarose (NGA) and allowed to solidify. Slides were placed in cold lysis solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 1% (w/v) sodium sarcosinate], with 1% (v/v) Triton X-100 and 10% (v/v) DMSO added freshly before each use, for 1.5 hrs. Slides were then placed in a horizontal gel electrophoresis tank (Horizon 20 25, GIBCO BRL, Life Technologies, Paisley, Scotland) containing fresh electrophoresis solution (1 mM EDTA, 300 mM NaOH) for 30 mins. Subsequently electrophoresis was carried out for 25 mins at 4°C with a current of 25 V (300 mA) using a compact power supply. After electrophoresis, the slides were washed three times with neutralizing buffer (0.4 M Tris, pH 7.5) at 4°C for 5 mins each. Slides were stained with ethidium bromide (20 µg/mL) and covered with coverslips. DNA integrity and damage were determined using the comet assay (Tice et al., 1990; Woods et al., 1999). A computer program Komet 5.5 image analysis software (Andor Technology, Belfast, Northern Ireland) was utilised to measure the level of DNA damage which was expressed as percentage tail DNA which represents the amount of DNA present in the tail. The longer the length of the comet then the greater the amount of DNA damage in the cell (as below).



Undamaged cell



Slightly damage cell



Extensively damaged cell

2.13. Statistical analysis

All experiments were repeated in quadruplicate. Results are presented as mean values ± SE. Statistical analysis was conducted using repeated measures ANOVA followed by Dunnett's or Tukey's test (Prism 4.0, GraphPad Inc, San Diego, CA, USA). Statistical significance was determined at $P < 0.05$ or $P < 0.01$.

3. Results and Discussion

3.1. Extraction procedure

In the present study methanol and water, both polar solvents, were used to extract bioactive compounds from the seaweed samples. Polar solvents efficiently extract a range of polar compounds such as sugar or protein attached polyphenols, tannins, salts, saponins, mucus, glycosides and organic acids (Cho et al., 2007). This extraction process was mainly aimed at extracting phlorotannins from the seaweed samples, although the extracts likely contain a range of additional polar compounds also.

3.2. Determination of Total Phenol Content (TPC)

Phenolic compounds can contribute considerably to the overall antioxidant capacity. The levels of phenols in the seaweed extracts ranged from 1.5 mg gallic acid equivalents (GAEq/gdw) in *L. hyperborea* to 4.5 mg GAEq/gdw in *A. nodosum* (Table 1). Similar phenolic concentrations were observed by Ganesan et al. (2008) in methanolic extracts of red seaweed which ranged from 1.5 mg GAEq/g (*Euchema kappaphycus*) to 4.1 GAEq/g (*Gracilaria edulis*). Devi et al. (2008) found much higher phenol levels in a variety of Indian sourced seaweeds where levels ranged from 47.5 GAEq/g (*Chondrococcus hornemanni*) to 616.3 GAEq/g (*Gellidela acerosa*). Chandini et al. (2008) on the other hand observed somewhat lower levels of phenols in the aqueous fractions of *Sargassum marginatum* and *Turbinaria conoides* which contained 0.29 and 0.86 mg GAE/g on a dry weight basis, respectively. Chew et al. (2008) reported that phenol content can vary quite considerably depending on the variety of seaweed; it was found that *Kappaphycus alvarezzi* contained 1.15 mg GAE/g while *Padina antillarum* contained levels of 24.30 mg GAE/g. Studies have shown that phenol content of seaweed is effected directly by sunlight and climate, therefore the phenol content of same seaweed species would differ from country to country depending on climate, for example a *F. serratus* sample taken from a warm country such as the Canary Islands would contain higher phenol levels than that of the same species harvested in Ireland (Flodin et al., 1999). The phenol levels in seaweed are also believed to be dependent on their location on the shore; seaweeds located in the highest intertidal zone are exposed to higher levels of UV-radiation and desiccation and the seaweed produces more phenols to protect against these stresses (Connan et al., 2007). Seaweeds

located lower down in the intertidal zone are submerged for longer periods of time and experience lower levels of stress and therefore require a lower concentration of phenols. Due to these factors the TPC of seaweed can vary significantly between different batches (Chew et al., 2008). In this study, there was some correlation seen between phenol levels and the location of the seaweed in the intertidal zone; *L. hyperborea* which is a lower shore species contained the lowest phenol concentration ($P < 0.05$) while conversely there was no significant difference in phenol content between *P. canaliculata*, *A. nodosum*, and *F. serratus* despite each being found in different intertidal zones.

3.3. Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP assay measures the ability of an antioxidant compound to reduce a ferric oxidant (Fe^{3+}) to a ferrous complex (Fe^{2+}) by electron-transfer, this indicates the capacity of the compound to reduce reactive species (Benzie & Strain, 1999; De et al., 2008). *F. vesiculosus* and *F. serratus* exhibited the highest FRAP activity at 109.8 and 113.4 μM AAEq/gdw, while the *L. hyperborea* displayed significantly lower ($P < 0.05$) FRAP activity at 25.6 μM AAEq/gdw (Table 1). Díaz-Rubio et al. (2008) measured the FRAP activities of a range of *F. vesiculosus* products and observed that FRAP activity differed quite significantly between extracts with activity ranging between 9.5 and 527.2 μM Trolox/g dw in a fucoidan extract and commercially available extract, respectively. Chew et al. (2008) observed a FRAP activity of between 0.56 and 15.7 mg GAE/g for extracts of *Kappaphycus alvarezzi* and *Padina antillarum*, respectively.

3.4. DPPH• (2,2-diphenyl-2-picrylhydrazyl hydrate) scavenging assay

The ability of a compound to scavenge DPPH radicals is dependent on their ability to pair with the unpaired electron of a radical (Park et al., 2004). *F. vesiculosus* and *A. nodosum* extracts scavenged DPPH radicals by 31.2% and 25.6%, respectively, while the other extracts exhibited lower scavenging abilities and *L. hyperborea* showed no scavenging effect. Chandini et al. (2008) observed that methanolic extracts of *S. marginatum* and *T. conoides* scavenged DPPH by 11% and 17.2 %, respectively, while an ethyl acetate fraction scavenged DPPH radical by 23.2%. Devi et al. (2008) reported that DPPH radical scavenging ability differed greatly between the different varieties and ranged between 5% and 72.5 % for *C.*

hornemanni and *G. acerosa*, respectively. Lu & Foo (2000) and Siriwardhana et al. (2003) reported a high correlation between DPPH scavenging and total phenol content of extracts. In the present study, although *A. nodosum, P. canaliculata, F. vesiculosus* and *F. serratus* contained somewhat similar phenol levels only *A. nodosum* and *F. vesiculosus* exhibited significant DPPH scavenging abilities whereas *L. hyperborea* which showed statistically lower levels of total phenols had no detectable DPPH scavenging capability. This suggests that the DPPH scavenging ability of extracts may also be dependent on the presence of small molecular compounds such as proteins, peptides and polysaccharides (Heo et al., 2005).

3.5. β-carotene Bleaching Assay (BCBA)

The bleaching of β–carotene in this assay is due to the presence of peroxyl free radicals which are created as a by-product of linoleic acid oxidation and this process can be altered if antioxidants present are able to compete with peroxyl radicals and thereby reduce/prevent the bleaching of β-carotene (Takada et al., 2006). All the seaweed extracts reduced β–carotene bleaching by at least 50% while *A. nodosum* and *F. vesiculosus* inhibited β–carotene bleaching by 76.3% and 71.2%, respectively. Mendiola et al. (2006) investigated the protective effect of a range of compounds extracted from *Spirulina platensis* via supercritical fluid extraction and found that β-carotene bleaching was reduced by between 1% and 97% depending on the extract. It is difficult to determine correlation between BCBA and phenol content due to the fact that the TPC assay measures the levels of both lipophilic and hydrophilic compounds whereas the BCBA is only affected by the presence of lipophilic compounds (Chew et al., 2008). The present study found no direct correlation between phenol levels and β–carotene bleaching inhibition.

3.6. Cell viability

Potential cytotoxic effects of the seaweed extracts at a range of concentrations between 0.55 and 5.5 mg/mL were investigated using the MTT assay. Cells remained viable for all seaweed extracts up to concentrations of 2 mg/mL (Figure 1). For subsequent experiments, the concentration of seaweed extract used did not exceed 2 mg/mL. The cytotoxic effects of seaweed extracts have been previously

attributed to their content of polysaccharides including ascophyllan, fucoidan and phlorotannins (Jiang et al., 2010; Yang et al., 2010).

Table 1

Total phenol content (TPC), ferric reducing antioxidant potential assay (FRAP), 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH•) scavenging assay and β-carotene bleaching assay (BCBA) of seaweed extracts.

	Ascophyllum nodosum[a]	*Laminaria hyperborea*[b]	*Pelvetia canaliculata*[c]	*Fucus vesiculosus*[d]	*Fucus serratus*[e]
TPC (mg gallic acid equivalent)	4.5 ± 0.5[b]	1.5 ± 0.5[a, c, e]	4.0 ± 0.5[b]	2.5 ± 0.5	4.0 ± 1.5[b]
FRAP (μM ascorbic acid)	81.0 ± 6.0	25.5 ± 4.0[d, e]	71.5 ± 10.5	109.5 ± 17.5[b]	113.5 ± 18.5[b]
DPPH (% radical scavenging)	25.5 ± 2.0[c, e]	nd	7.5 ± 2.0[a, d]	31.5 ± 3.5[c, e]	5.5 ± 1.5[a, d]
BCBA (% protection)	76.5 ± 3.5[b, c]	50.0 ± 5.5[a, d]	54.0 ± 1.2[a, d]	71.5 ± 3.5[b, c]	62.5 ± 2.0

([a-e]) denotes a significant difference between mean values, repeated measures ANOVA followed by Tukey's post-test. n= 4 independent experiments.

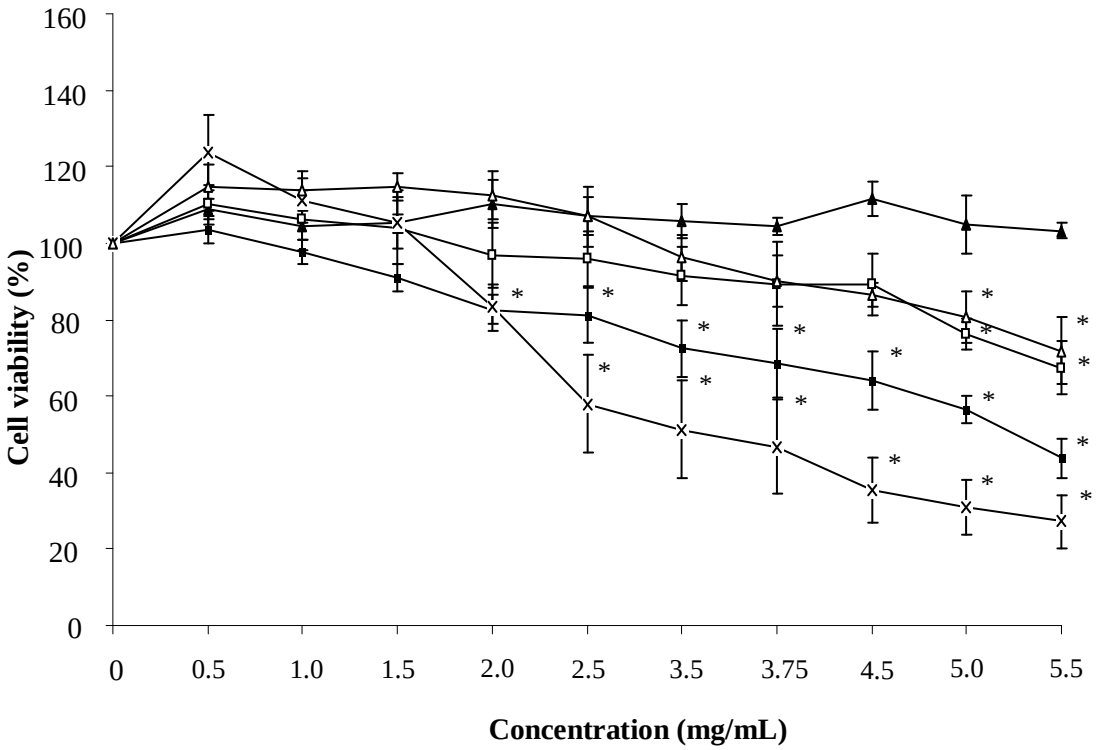


Figure 1. Viability of Caco-2 cells following addition of increasing levels (mg/mL) of *Ascophyllum nodosum* (-■-), *Laminaria hyperborea* (-▲-), *Pelvetia canaliculata* (-□-),*Fucus vesiculosus* (-×-) and *Fucus serratus* (-Δ-). Data are the means of four independent experiments, with standard errors represented by vertical bars. * Mean value was significantly different from that of control cells ($P < 0.05$). n= 4 independent experiments.

3.7. Enzymatic antioxidant activity

Catalase is a porphyrin-containing enzyme which catalyzes the decomposition of hydrogen peroxide to water and oxygen (Aruoma et al., 1998). The addition of 200 μM H_2O_2 to Caco-2 cells increased CAT activity by 25% compared to the unchallenged control (Table 2) which was expected due to the CAT inducing activity of H_2O_2 and was similarly reported by Wijeratne et al. (2005). The addition of seaweed extracts had no significant effect ($P < 0.05$) on the CAT activity of H_2O_2-challenged cells. This suggests that the seaweed extracts lack the *in-vitro* H_2O_2 scavenging (Heo et al., 2005) and catalase enhancing (Kang et al., 2005) activities previously reported in seaweed extracts. Superoxide dismutases are a class of enzymes which catalyze the dismutation of superoxide into oxygen and hydrogen peroxide (Aruoma et al., 1998). The addition of 200 μM H_2O_2 to Caco-2 cells reduced SOD activity by 35% compared to the unchallenged control (Table 2). A similar trend was reported by Wijeratne et al. (2005) where a 47% reduction in SOD activity was observed under similar experimental conditions. The reduction in SOD activity may be due to an interaction between H_2O_2 and a copper moiety in the SOD molecule (Bray et al., 1974; Symonyan & Nalbandyan, 1972; Jhonson & MacDonald, 2004). All seaweed extracts offered significant protection ($P < 0.05$) against H_2O_2-mediated SOD reduction. *P. canaliculata* extract provided the greatest protection of the five seaweed extracts examined. Wijeratne & Cuppett (2007) investigated the protective effect of two oil extracts from Rosemary in oleic acid hydroperoxide (OAHPx)-oxidised Caco-2 cells and it was observed that both extracts protected fully against SOD depletion. A study by Veena et al. (2007) investigated the effect of a *F. vesiculosus* extract on the enzymatic antioxidant status in hyperoxaluria affected rat kidneys and found that the extract helped to protect against CAT and SOD reduction by 73% and 100%, respectively, however the damaging mechanism of hyperoxaluria is different to that of H_2O_2.

3.8. Reduced Glutathione (GSH) activity

GSH is a tripeptide consisting of glutamine, cysteine and glycine and is a key component in the non-enzymatic antioxidant mechanism of cells (Viňa, 1990). In the present study, it was found that each of the seaweed extracts caused a significant increase ($P < 0.05$) in GSH activity in Caco-2 cells with *F. serratus* increasing GSH levels by approximately 8 nmol GSH eq/mg protein, compared to the control (Table

3). Using a similar experimental design, Aherne et al. (2007) reported that an extract of sage increased GSH activity by approximately 43% in Caco-2 cells. Ngo et al. (2008) observed that an extract from crab chitin significantly increased the GSH activity of mouse macrophage cells.

Table 2

The effect of seaweed extracts on the antioxidant status of Caco-2 cells challenged with 200 μM H_2O_2 for 30 mins.

Seaweed extract	Catalase activity (% of control)	SOD activity (% of control)
Control[a]	100.0	100.0
H_2O_2 control[b]	125.1 ± 6.1	64.9 ± 5.9 [c, d, e, f, g]
Ascophyllum nodosum[c]	135.5 ± 6.7	89.5 ± 2.4 [b]
Laminaria hyperborea[d]	116.9 ± 7.3	92.2 ± 1.5 [b]
Pelvetia canaliculata[e]	138.7 ± 9.6	97.4 ± 3.7 [b]
Fucus vesiculosus[f]	131.9 ± 3.0	89.0 ± 2.7 [b]
Fucus serratus[g]	137.4 ± 8.5	83.1 ± 9.2 [b]

([a-g]) denotes a significant difference between mean values, repeated measures ANOVA followed by Dunnett's post-test. n= 4 independent experiments.

Table 3

The effect of seaweed extracts on the reduced glutathione activity in Caco-2 cells.

Seaweed extract	Reduced glutathione content (nmol GSH eq/mg protein)
Control[a]	21.9 ± 0.8 [b, c, d, e, f]
Ascophyllum nodosum[b]	27.6 ± 1.2 [a]
Laminaria hyperborea[c]	26.5 ± 0.6 [a]
Pelvetia canaliculata[d]	26.1 ± 0.3 [a]
Fucus vesiculosus[e]	27.3 ± 1.0 [a]
Fucus serratus[f]	30.1 ± 1.6 [a]

([a-f]) denotes a significant difference between mean values, repeated measures ANOVA followed by Dunnett's post-test. n= 4 independent experiments.

3.9. Determination of DNA damage

The comet assay can be utilised to measure various forms of DNA damage including single-strand breaks, double-strand breaks and incomplete repair sites (Tice et al., 2000). In the present study, the addition of 50 μM H_2O_2 to Caco-2 cells caused an increase in DNA damage by approximately 52.5% compared to the unchallenged control (Figure 2), similar to that previously measured in our laboratory (Aherne & O'Brien, 1999, 2000; Aherne et al., 2007). The H_2O_2-mediated DNA damage is likely due to an interaction between H_2O_2 and ferrous ions which forms hydroxyl radicals which can cause DNA damage (Barbouti et al., 2001). These ferrous ions are result of the superoxide radical-mediated reduction of ferric ions (Starke and Farber, 1985). In the present study there was a significant decrease ($P < 0.05$) in DNA damage in the cells supplemented with the *F. vesiculosus* and *F. serratus* extracts which protected by 10.5% and 13.5%, respectively. Previous studies have demonstrated a greater DNA protective effect for seaweed extracts. Heo et al. (2005) which found that enzymatic extracts of *S. horneri* reduced H_2O_2-induced DNA damage by 50% while Gamal-Eldeen et al. (2009) observed that three extracts from *Sargassum latifolium* reduced DNA damage in methyl methane sulfonate-challenged human lymphocytes by between 24.5% and 64.25%. An extract from *Grateloupia filicina* exhibited a more potent cytoprotective effect in rat lymphocytes by reducing H_2O_2-induced damage by 69% (Athukorala et al., 2005). In the present study it was observed that *F. vesiculosus* and *F. serratus* extracts exhibited both the greatest FRAP values and GSH enhancing activities while also displaying the most protective effect against H_2O_2-induced DNA damage. Therefore, it is possible that the cytoprotective capacities of the *F. vesiculosus* and *F. serratus* extracts may be attributed to their ferric ion binding or GSH enhancing abilities, or a combination of both activities.

Conclusion

In the study the *F. vesiculosus* and *F. serratus* extracts were seen to be the most effective extracts in terms of antioxidant activity. The *F. serratus* extract contained the highest phenolic levels while both extracts were found to be powerful ferric radical scavengers. The *F. vesiculosus* extract was the most effective extract at preventing β–carotene bleaching and scavenging DPPH radicals. In terms of cellular

activity, both extracts protected against H_2O_2-mediated SOD reduction while also exhibiting potent GSH activity enhancing abilities. Both extracts provided the best protection against H_2O_2-mediated DNA damage in Caco-2 cells. Pending further analysis and experimentation, these natural seaweed extracts may have potential applications for use in the food and pharmaceutical industries. Both *F. vesiculosus* and *F. serratus* are abundant on the Irish coast making them a sustainable source of natural antioxidants.

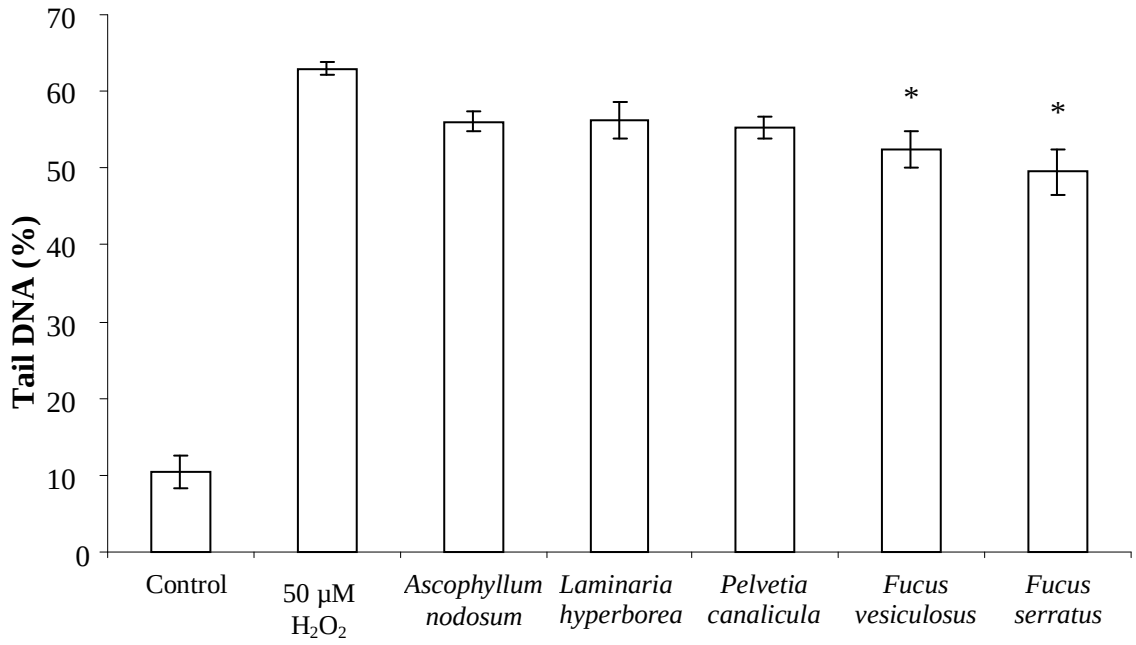


Figure 2. DNA damage in Caco-2 cells following pre-treatment with or without seaweed extracts (100 µg/mL) for 24 hrs then exposed to 50 µM -H_2O_2 for 30 mins at 37°C. DNA damage was assessed by the comet assay (explained earlier) in which % tail DNA was measured. Data are the means of four independent experiments, with SEM represented by vertical bars. Statistical analysis was by repeated measures ANOVA followed by the Dunnett's test. Mean value was significantly different from that for H_2O_2-challenged cells ($P < 0.05$). n = 4 independent experiments.

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CHAPTER 3

Assessment of the ability of seaweed extracts to protect against hydrogen peroxide and *tert*-butyl hydroperoxide induced cellular damage in Caco-2 cells.

Based on:

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Abstract

The ability of brown seaweed extracts, *Ascophyllum nodosum*, *Laminaria hyperborea*, *Pelvetia canaliculata*, *Fucus vesiculosus* and *Fucus serratus* to protect against *tert*-butyl hydroperoxide (*tert*-BOOH) induced stress in Caco-2 cells was investigated. Oxidative stress was determined by measuring alteration in the enzymatic activity of catalase (CAT) and superoxide dismutases (SOD) and cellular levels of glutathione (GSH). *L. hyperborea*, *P. canaliculata* and *F. serratus* significantly protected against *tert*-BOOH induced SOD reduction but did not protect against the reduction in CAT activity or the increased cellular levels of GSH. The ability of *F. serratus* and *F. vesiculosus* to protect against H_2O_2 and *tert*-BOOH induced DNA damage was also assessed. The DNA protective effects of the two seaweed extracts was compared to those of three metal chelators; deferoxamine mesylate (DFO), 1, 10-phenanthroline (o-phen) and 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (BAPTA-AM). *F. serratus* and *F. vesiculosus* significantly protected ($P < 0.05$) against H_2O_2 (50 μM) induced DNA damage but not *tert*-BOOH induced damage.

1. Introduction

Seaweed consumption has been associated with a range of health benefits such as anticancer, immunomodulatory, antiviral, antibacterial and antioxidant activities which are attributed to seaweed constituents such as sulphated polysaccharides, carrageenans, fucoidans, terpenoids and polyunsaturated fatty acids (Smit, 2004). In addition, studies suggest that seaweed extracts prevent lipid peroxidation and therefore may be a useful alternative to synthetic antioxidants in food systems such as meat, fish and dairy products (Wang et al., 2009) while also providing beneficial effects to human health. Phlorotannins are phenolic compounds found in brown seaweed which have demonstrated protective effects against oxidant induced cellular antioxidant enzyme depletion and DNA damage in human cells (Heo et al., 2009; O'Sullivan et al., 2011).

Hydrogen peroxide (H_2O_2) and *tert*-butyl hydroperoxide (*tert*-BOOH), both physiologically relevant oxidants, have been used to generate an established model of oxidative stress in a number cell lines (Ozden et al., 2009; Valls-Bellés et al., 2004). H_2O_2 is mainly produced as a mitochondrial by-product and also released by phagocytic activity. *tert*-BOOH is an synthetic form of lipid hydroxide naturally produced in the body and in food by the oxidation of polyunsaturated fatty acid residues of phospholipids. The mechanism of H_2O_2 and *tert*-BOOH induced oxidative damage is dependent on the presence of transition metals such as copper and iron. H_2O_2 reacts with ferrous ions via the Fenton reaction to form hydroxyl radicals which can cause base modifications, sister chromatid exchange, single and double strand breaks in DNA while copper may also have a role in hydroxyl radical production (Barbouti et al., 2001). Hydroxyl radicals can also damage calcium regulating proteins leading to excessive calcium levels which can result in endonuclease activation and single strand breaks in DNA (Barbouti et al., 2001). *tert*-BOOH reacts with copper and iron to produce *tert*-butoxyl or *tert*-butylperoxyl radicals causing lipid peroxidation and the generation of a wide range of radical species which may cause substantial damage both to the cell and its constituent DNA (Kennedy et al., 1992).

Compounds with the capacity to sequester metal ions can protect against H_2O_2- and *tert*-BOOH-induced cell damage. A number of studies have demonstrated a correlation between metal chelation (binding) and antioxidant potency (Sestili et al., 2002). Polyphenols have been shown to reduce or prevent H_2O_2-mediated DNA

damage in *E. coli* cells by chelating iron (Perron et al., 2008). In addition, polyphenols such as quercetin and rutin have been shown to reduce *tert*-BOOH-induced damage in Caco-2 cells due to their metal chelating abilities (Aherne & O'Brien, 2000). Green seaweed such as *Halimeda macroloba* can contain high levels of polyphenols such as epigallocatechin and catechol, both of which have exhibited potent iron binding and DNA protective capabilities against H_2O_2-induced damage (Yoshie et al., 2002; Perron et al., 2008). The phlorotannin eckol, which is isolated from brown seaweed, protects against H_2O_2-induced DNA damage by a mechanism which is believed to involve an increase in both the protein expression and activity of catalase, resulting in an enhanced break down of H_2O_2 (Kang et al., 2005). Seaweed also contains significant levels of vitamin C, vitamin E and carotenoids, all of which have been shown to protect against oxidant induced DNA damage (Cemelia et al., 2009).

In a previously reported study, extracts of *Fucus vesiculosus* and *Fucus serratus* reduced H_2O_2-mediated DNA damage while *Pelvetia canaliculata* extract provided almost complete protection against H_2O_2-mediated SOD depletion in Caco-2 cells (O'Sullivan et al., 2011). The objective of the present study was to establish if methanolic extracts of brown seaweeds (*Ascophyllum nodosum, Laminaria hyperborea, P. canaliculata, F. vesiculosus* and *F. serratus*) can protect against the *tert*-BOOH induced depletion in superoxide dismutase (SOD) and catalase activity and increased cellular glutathione levels in Caco-2 cells. In addition, the ability of extracts of *F. serratus* and *F. vesiculosus* to protect against H_2O_2 and *tert*-BOOH induced DNA damage was examined. The DNA protective effects of seaweed extracts were compared to those of three metal chelators; deferoxamine mesylate (DFO) which chelates iron, 1, 10-phenanthroline (o-phen) which chelates ferrous and copper ions, and 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (BAPTA-AM) which chelates calcium. Such chelators have been used in several studies to investigate mechanisms involved in DNA damage (Guidarelli et al., 1997; Aherne & O'Brien. 2000; Barbouti et al., 2001). DNA damage was measured using the comet assay.

2. Materials and Methods

2.1. Materials

Human colon adenocarcinoma cells (Caco-2) were acquired from the European Collection of Animal Cell cultures (Salisbury, UK), Foetal Bovine Serum (FBS) was purchased from Invitrogen (Paisley, Scotland). Cell culture plastics were purchased from Cruinn Diagnostics (Greiner Bio-One, Frickenhausen, Germany). Calbiochem SOD Assay Kit II was purchased from Merck Chemicals Ltd. (Nottingham, UK). All other cell culture chemicals and reagents were purchased from Sigma Chemical Co. (Dublin, Ireland).

2.2. Preparation of extracts and chelators

Seaweed samples were collected and prepared as previously described in Chapter 2. The seaweed extracts were reconstituted in 10 ml distilled water resulting in a final concentration of 10 mg/ml for addition to cells. The metal chelators DFO, o-phen and BAPTA-AM were dissolved in cell culture media, DMSO and H_2O, respectively and were added to the cells at concentrations ranging from 2.5-15 mM, 25-1000 μM and 0.5-10 μM, respectively.

2.3. Cell culture

Human colon adenocarcinoma, Caco-2 cells were grown in antibiotic-free Dulbecco's modified Eagle's medium (DMEM) supplemented with FBS (10% v/v) and non-essential amino acids (1% v/v). Cells were incubated in an atmosphere of CO_2–air (5:95, v/v) at 37°C. Caco-2 cells were plated at a density of 1×10^5 or 2×10^5 cells/ml for the comet and antioxidant assays, respectively.

2.4. Antioxidant Assays

Caco-2 cells were supplemented with 100 μg/ml seaweed extracts for 24 hrs in 6-well plates with a final volume of 2 ml media containing reduced FBS (2.5% v/v). The cells were subsequently incubated with *tert*-BOOH for 30 mins in serum free media. Cells were harvested and homogenised to release intracellular enzymes for the determination of the cellular antioxidant status. In the superoxide dismutases (SOD) assay, cells were exposed to 1000 μM *tert*-BOOH or 200 μM H_2O_2 and SOD activity was determined using a Calbiochem SOD Assay Kit II (Merck Chemicals

Ltd., Nottingham, UK). Catalase (CAT) activity was determined following exposure of cells to 100 μM *tert*-BOOH using a Calbiochem catalase colorimetric activity kit (Merck Chemicals Ltd., Nottingham, UK). The glutathione (GSH) content was measured according to the method of Hissin & Hilf (1976) following exposure to 2000 μM *tert*-BOOH. In short, perchloric acid (15%, v/v) was added to the cell sonicates and samples were centrifuged at 14000 rpm for 30 mins at 4°C. The final GSH assay mixture contained 100 μl sample, 1.8 ml sodium phosphate-ethylenediamine tetraacetic acid buffer, pH 8, and 100 μl o-phthaldialdehyde (1 mg/ml). Fluorescence was detected at 430 nm following excitation at 360 nm using a Spectrofluor plus platereader (Tecan, Männedorf, Switzerland).

SOD, catalase and GSH were expressed relative to the protein content (mg), as determined by the bicinchoninic acid (BCA) method (Smith et al., 1985). SOD and catalase were expressed as units mg^{-1} protein where one unit of SOD is defined as the amount of enzyme needed to induce 50% dismutation of the superoxide radical and one unit of catalase activity is defined as the amount of enzyme required to produce 1 nM formaldehyde min^{-1} at 25 °C. GSH were expressed as nmol mg^{-1} protein.

2.5. Determination of DNA damage (Comet assay)

Caco-2 cells were supplemented with 100 μg/ml seaweed extracts for 24 hrs in 6-well plates with a final volume of 2 ml media, containing reduced FBS (2.5% v/v). Following incubation, the cells were treated with or without 50 μM H_2O_2 or 200 μM *tert*-BOOH for 30 mins. Cells were harvested, embedded in low melting point (LMP) agarose and placed on microscope slides. The comet assay was conducted as previously described in Chapter 2. Komet 5.5 image analysis software (Andor Technology, Belfast, Northern Ireland) was used to measure the level of DNA damage which was expressed as percentage tail DNA.

2.6. Statistical analysis

Data represent the means of four independent experiments ± standard error (SE), which are represented by vertical bars. Statistical analysis was conducted using repeated measures ANOVA followed by Dunnett's or Tukey's test (Prism 4.0, GraphPad Inc, San Diego, CA, USA). The level of statistical significance was taken at $P < 0.05$ or $P < 0.01$.

3. Results and Discussion

3.1. Enzymatic antioxidant activity

Seaweed extracts contain bioactive compounds such as phlorotannins. The bioactive compounds investigated in the present study were extracted from the seaweed samples using methanol and water which has previously been demonstrated as an effective method for the extraction of phlorotannins. Extracts may also contain salts, saponins, mucus, glycosides and organic acids (Hagerman, 1988; Cho et al., 2007).

The first objective was to determine if the seaweed extracts protected against *tert*-BOOH induced oxidative stress in Caco-2 cells. The optimal concentration of *tert*-BOOH required to induce a significant alteration in the antioxidant status (SOD, catalase, GSH) of the cells was determined in a preliminary experiment (data not shown).

The addition of 1000 µM *tert*-BOOH to Caco-2 cells reduced the SOD activity to 73.9% that of the control (Table 1). The addition of 100 µg/ml of *L. hyperborea* ($P < 0.05$), *P. canaliculata* ($P < 0.05$) and *F. serratus* ($P < 0.01$) to Caco-2 cells significantly protected against the *tert*-BOOH mediated depletion in SOD activity, increasing activity from 73.9% to 100.1%, 97.0% and 108.0%, respectively. Extracts of *A. nodosum* and *F. vesiculosus* did not significantly protect against the *tert*-BOOH mediated reduction in SOD activity. In a previously reported study, all seaweed extracts examined in the present study significantly protected against H_2O_2 mediated depletion of SOD activity (O'Sullivan et al., 2011) suggesting that seaweed extracts are more effective at protecting against the mechanisms of H_2O_2-induced SOD depletion than that of *tert*-BOOH.

A possible mechanism for the protective effects exhibited by the seaweed extracts against H_2O_2 and *tert*-BOOH induced SOD depletion may be copper chelation as copper chelation has demonstrated a protective effect against oxidant induced SOD fragmentation (Kang et al., 2002). Therefore, the copper chelator, o-phen, was used in an attempt to determine if the chelation of copper was effective against the SOD depletion induced by *tert*-BOOH and H_2O_2. However, no significant protective effect on SOD activity in the presence of the copper chelator (Figure 1) was reported and therefore H_2O_2 / *tert*-BOOH-induced depletion of SOD activity is unaffected by copper chelation. At concentrations of 500 and 1000 µM o-phen appeared to accentuate *tert*-BOOH-induced SOD depletion and this effect could not be fully

explained, however, it is postulated that at higher concentrations o-phen increased the SOD depleting effect of *tert*-BOOH. Various polyphenol compounds have demonstrated ability to upregulate mRNA expression of SOD (Yeh et al., 2009) which may account for the increase in SOD activity observed in the seaweed extract treated cells (Table 1).

The addition of 100 µM *tert*-BOOH to Caco-2 cells decreased CAT activity to 80% of the unchallenged control (Table 1). There was no significant difference in the catalase level of cells exposed to *tert*-BOOH either in the presence or absence of the seaweed extracts investigated in the present study (Table 1). The glutathione cycle is the principal mechanism involved in removing hydroperoxides from the body and previous studies have demonstrated that the intracellular content of GSH may be increased under conditions of oxidative stress (Wijeratne & Cuppett, 2006). The addition of 2000 µM *tert*-BOOH to Caco-2 cells resulted in a GSH increase of approximately 32%. The pre-incubation of Caco-2 cells with seaweed extracts had no significant effect ($P < 0.05$) on the *tert*-BOOH mediated increase in the GSH levels (Table 1).

3.2. DNA damage

Seaweed extracts, *F. serratus* and *F. vesiculosus*, were selected for further investigation based on their effectiveness against the DNA damaging effects of H_2O_2, investigated by O'Sullivan et al. (2011). The present study compared the seaweed extracts with metal chelators in their ability to protect against DNA damage induced by both H_2O_2 and *tert*-BOOH which have previously been shown to induce DNA damage in a range of cell lines (Aherne & O'Brien, 1999). Research conducted to date would suggest that H_2O_2-mediated DNA damage results from the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) by superoxide radicals, the ferrous ions then react with H_2O_2 to form hydroxyl radicals which cause DNA damage (Valko et al., 2005). *tert*-BOOH mediated oxidative stress occurs via a metal activated decomposition of the compound which leads to an alteration in cellular calcium levels, lipid peroxidation and the generation of additional radicals, resulting in substantial damage to cell integrity (Kennedy et al., 1992; Halliwell & Gutteridge, 1999; Li et al., 2008).

In the present study, a dose response was carried out for the three chelators, (DFO, o-phen and BAPTA-AM) to determine their protective effect against both H_2O_2 and

tert-BOOH–induced DNA damage in the Caco-2 cell line (Figure 2). The addition of 50 μM H_2O_2 and 200 μM *tert*-BOOH to Caco-2 cells increased the percent tail DNA from a baseline control level of 14.5% to 55.2% and 33.4%, respectively, as measured by the comet assay. All three metal chelators demonstrated an ability to significantly protect against *tert*-BOOH-induced DNA damage while DFO and o-phen significantly protected against H_2O_2-induced DNA damage. The calcium chelator, BAPTA-AM, has previously been shown to reduce DNA damage in H_2O_2-challenged Jurkat cells by approximately 75% (Barbouti et al., 2001) however BAPTA-AM (10 μM) had no significant effect against H_2O_2-mediated damage in the Caco-2 cell line (Figure 2C). o-Phen provided nearly complete protection against both H_2O_2 and *tert*-BOOH-mediated DNA damage at the highest concentration (500 μM). Extracts of the seaweeds, *F. serratus* ($P < 0.05$) and *F. vesiculosus* ($P < 0.01$), reduced H_2O_2-induced DNA damage to approximately 45.7% and 42.0% tail DNA, respectively, from a control level of 55.2% tail DNA in H_2O_2-treated cells (Figure 3). However, neither seaweed extract significantly protected against *tert*-BOOH induced DNA damage (Figure 3).

A study conducted by Kruszewski et al. (2008) found that the DNA damage induced by H_2O_2 was more dependent on iron status than DNA damage induced by *tert*-BOOH in neonatal pig lymphocytes. Therefore, it is possible that the DNA protective effects of the seaweed extracts are related to iron chelation as they were more effective against the DNA damaging effects of H_2O_2 than *tert*-BOOH. Seaweed extracts have previously demonstrated an ability to chelate ferrous iron in a non-cell based model (Wang et al., 2009), the authors attributed the chelating ability more to the protein and peptide fraction rather than the phlorotannin fraction. A study by Ahn et al. (2007) suggested an alternative mechanism whereby the phlorotannins eckol, phloroglucinol and dieckol provided DNA protection against H_2O_2 damage by enhancing cellular catalase activity which in turn neutralises the damaging effects of H_2O_2 (Kang et al, 2005). Another hypothesis is that the seaweed extracts may upregulate catalase expression in the cells similar to the effect green tea polyphenols demonstrated in rat heart muscle (Negishi et al., 2004).

Extracts from sea-buckthorn (*Hippophae rhamnoides* L.) have been previously found to reduce intracellular calcium levels and *tert*-BOOH-mediated DNA damage (Geetha et al., 2009). The calcium chelator BAPTA-AM reduced *tert*-BOOH-mediated DNA damage however the seaweeds exhibited no such effect and we must

conclude that they did not possess sufficient calcium chelating ability.

Table 1: The effect of seaweed extracts on the antioxidant status of Caco-2 cells challenged with *tert*-BOOH for 30 mins

	SOD activity (% Control)	CAT activity (% Control)	GSH content (% Control)
tert-BOOH	73.9 ± 2.1	79.7 ± 3.3	132.3 ± 2.8
Ascophyllum nodosum + tert-BOOH	90.1 ± 2.1	73.9 ± 9.6	108.4 ± 14.3
Laminaria hyperborea + tert-BOOH	100.1 ± 7.1*	75.5 ± 1.9	126.9 ± 11.1
Pelvetia canaliculata + *ter*t-BOOH	97.0 ± 6.1*	51.5 ± 20.2	115.2 ± 13.2
Fucus vesiculosus + *tert*-BOOH	93.8 ± 8.0	83.3 ± 7.0	111.6 ± 20.4
Fucus serratus + *tert*-BOOH	108.0 ± 8.4**	86.6 ± 3.9	128.0 ± 9.3

Caco-2 cells were pre-incubated with 100 µg/ml seaweed extracts for 24 hrs. Superoxide dismutase activity was assessed following 30 mins incubation with 1000 µM *tert*-BOOH. Catalase activity of the cells was assessed following 30 mins incubation with 100 µM *tert*-BOOH. Glutathione content was assessed following 30 mins incubation with 2000 µM *tert*-BOOH. * $P < 0.05$, ** $P < 0.01$ significantly different from *tert*-BOOH-treated cells, ANOVA followed by Dunnett's post-test. Data represent 4 individual experiments ± SE.

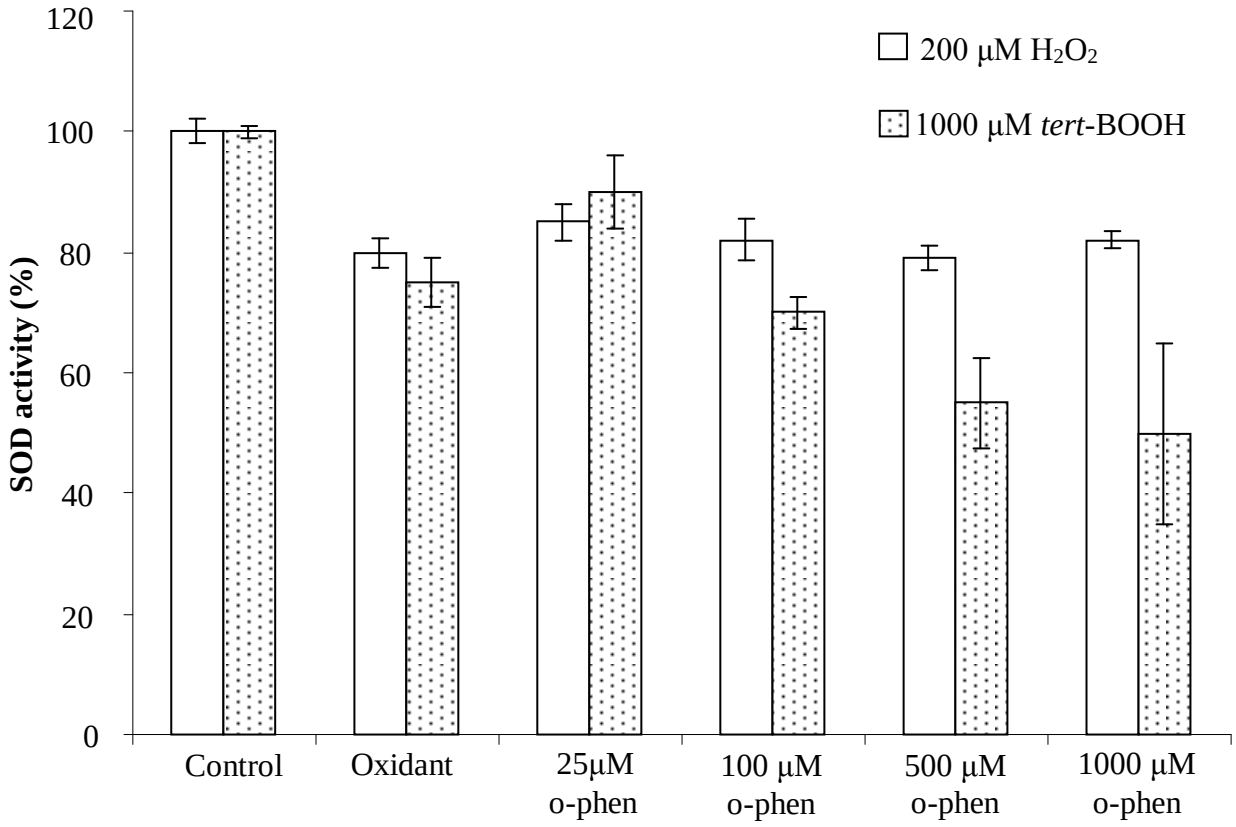


Figure 1: SOD activity, expressed as a percentage of the control value. Caco-2 cells were exposed to 200 μM H_2O_2 [□] or 1000 μM *tert*-BOOH [⁘] for 30 mins following a preincubation with metal ion chelator, 1,10-phenanthroline for 24 hrs. Statistical analysis was by repeated measures ANOVA followed by the Dunnett's test.

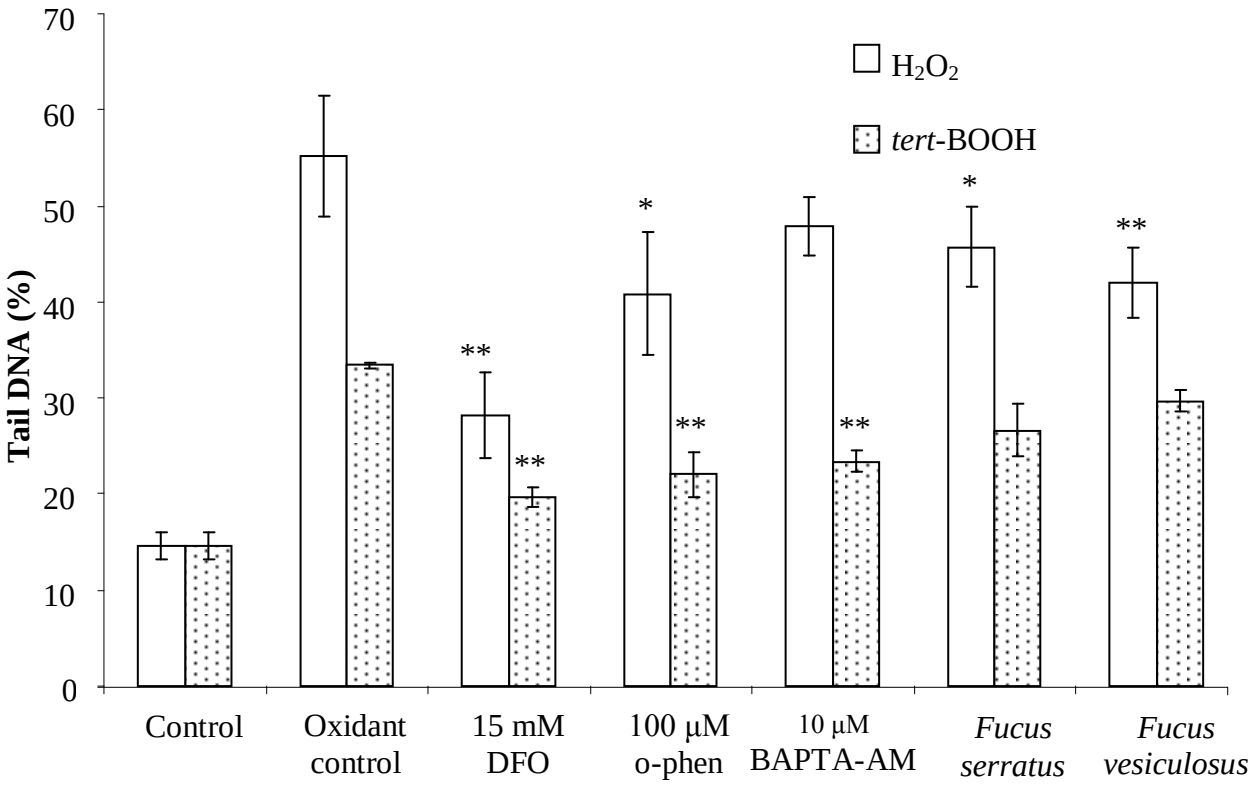


Figure 2: DNA damage in Caco-2 cells, expressed as percent tail DNA. Cells were incubated with metal chelators, 15 mM desferoxamine mesylate, 100 µM 1,10-phenanthroline, 10 µM BAPTA-AM for 30 mins or seaweed extracts *Fucus serratus* or *Fucus vesiculosus* for 24 hrs, media was removed and replaced with media containing 50 µM H_2O_2 [□] or 200 µM *tert*-BOOH [⬚] for a further 30 mins. Statistical analysis was by repeated measures ANOVA followed by the Dunnett's test. * Mean value was significantly different from that for H_2O_2 or *tert*-BOOH -challenged cells ($P < 0.05$). ** Mean value was significantly different from that for H_2O_2 or *tert*-BOOH -challenged cells ($P < 0.01$).

4. Conclusions

Seaweed extracts, *L. hyperborea*, *P. canaliculata and F. serratus*, protected against *tert*-BOOH-mediated reduction in SOD activity. The extracts did not ameliorate the decrease in catalase activity or cell glutathione levels induced by *tert*-BOOH. *F. vesiculosus* and *F. serratus* protected against H_2O_2 but not *tert*-BOOH-induced DNA damage in Caco-2 cells under the conditions of the present study. The seaweed extracts protected against H_2O_2-induced DNA damage to a degree similar to that of 100 µM of ferrous/copper chelator, o-phen, however further research is necessary to establish if the antioxidant effects of seaweed extracts result from metal chelation.

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CHAPTER 4

The effect of solvents on the antioxidant activity in Caco-2 cells of Irish brown seaweed extracts prepared using accelerated solvent extraction (ASE®).

Based on:

O'Sullivan, A.M., O'Callaghan, Y.C., O'Grady, M.N., Hayes, M., Kerry, J.P. & O'Brien, N.M. (2012). The effect of solvents on the antioxidant activity in Caco-2 cells of Irish brown seaweed extracts prepared using accelerated solvent extraction (ASE®). *Journal of Functional Foods* (DOI number: 10.1016/j.jff.2013.02.007).

Abstract

The potential antioxidant activities of extracts from *Ascophyllum nodosum* (*AN*), *Fucus vesiculosus* (*FV*) and *Fucus serratus* (*FS*) harvested from the west coast of Ireland and prepared using different solvents were assessed in a Caco-2 cell model. The extracts were prepared using 100% H_2O (AN_{100}, FV_{100}, FS_{100}), 60% ethanol: 40% H_2O (AN_{60e}, FV_{60e}, FS_{60e}), 80% ethanol : 20% H_2O (AN_{80e}, FV_{80e}, FS_{80e}) or 60% methanol : 40% H_2O (AN_{60m}, FV_{60m}, FS_{60m}) in combination with an accelerated solvent extraction (ASE®) technique. The effect of the extracts on the antioxidant status of the Caco-2 cells was determined by measuring catalase (CAT) activities and reduced glutathione (GSH) content. Superoxide dismutase (SOD) activity was assessed in both oxidant challenged and unchallenged cells. The protective effects of the extracts against H_2O_2 and *tert*-BOOH -induced DNA damage were assessed using the comet assay. The addition of the ASE® extracts did not significantly alter the cellular antioxidant status of the cells. AN_{100} and AN_{80e} significantly protected ($P < 0.05$) against H_2O_2-induced DNA damage and AN_{60e}, AN_{80e}, FS_{100}, FS_{80e} and FV_{60m} protected against *tert*-BOOH -induced DNA damage. Extracts prepared from *AN*, particularly those extracts prepared using 80% aqueous ethanol, appeared to have stronger antioxidant potential compared to other extracts based on their ability to protect against oxidant-induced DNA damage.

1. Introduction

Seaweed in the human diet has been associated with a range of health benefits such as antiviral, antioxidant, immunomodulatory and cardiovascular protective effects *in-vivo* (Burtin, 2003). The bioactive components of seaweeds have been identified as carrageenan, sulphated polysaccharides, fucoidans, terpenoids and polyunsaturated fatty acids (Smit, 2004). The predominant antioxidant compounds found in brown seaweed are phlorotannins (Kang et al., 2006). The primary function of phlorotannins is to protect seaweed from the harmful effects of UV exposure (Pavia et al., 1997). Phlorotannins have been reported to protect against UV-B radiation-induced DNA damage in human skin fibroblasts (Heo et al., 2009) and H_2O_2-induced DNA damage in monkey kidney fibroblasts (Heo & Jeon, 2009).

The profile of compounds extracted from material is dependent on the polarity of the solvent. The solvent or combination of solvents used for extraction influences the type, yield and potency of the antioxidant compounds. Solvents such as methanol and ethanol are believed to be more efficient than water at extracting antioxidant compounds from plant material because they break down cell walls and other structures in plants more effectively (Lapornik et al., 2005). Jakopič et al. (2009) reported that methanol was more efficient in extracting polyphenols from green walnut fruits than ethanol and also that the antioxidant profiles of the extracts varied significantly depending on the extraction solvent. In macroalgae (seaweed) Cho et al. (2007) noted that water produced a higher extraction yield (weight) of extract from *Sargassum siliquastrum*, a brown seaweed, but an ethanol extraction resulted in higher phenol levels. Heo et al. (2005) compared the effect of solvent and temperature on the type of bioactive compounds extracted from a large variety of Korean seaweeds and reported that the resulting extracts varied very significantly in terms of polyphenol and antioxidant profile. The methanolic and aqueous extracts of the examined seaweeds contained antioxidant compounds that strongly scavenged ROS such as superoxide anions, hydroxyl radicals, hydrogen peroxide and DPPH free radicals.

The antioxidant potential of several seaweed extracts have been assessed at a cellular level using *in-vitro* cell models (Ahn et al., 2007; Kang et al., 2005; Kang et al., 2006). Shukla et al. (2009) investigated the antioxidant effects of spirulina extracts and found that an aqueous extract exhibited significantly better protective effects against *tert*-BOOH induced cytotoxicity and oxidative stress in comparison to an

ethanolic extract, in C6 glioma cells. Previous studies have found that extracts from seaweeds protect against oxidant-induced DNA damage in a range of cell models (Athukorala et al., 2005; Ahn et al., 2007; Heo & Jeon, 2009).

Methanolic extracts of brown seaweeds, *Ascophyllum nodosum*, *Fucus vesiculosus* and *Fucus serratus* have previously been shown to exhibit antioxidant effects *in-vitro* (O'Sullivan et al., 2011; O'Sullivan et al., 2012) and therefore these seaweeds were selected for further investigation in the present study. The objective of this study was to compare four solvent systems for their ability to extract antioxidant compounds from *Ascophyllum nodosum (AN)*, *Fucus vesiculosus (FV)* and *Fucus serratus* (*FS)* to determine the most effective solvent for the optimal extraction of antioxidant compounds. The solvent systems were 100% water, 60% ethanol (EtOH) (v/v), 80% EtOH (v/v) and 60% methanol (MeOH) (v/v) and the resulting extracts were termed AN_{100}, FV_{100}, FS_{100}, AN_{60e}, FV_{60e}, FS_{60e}, AN_{80e}, FV_{80e}, FS_{80e} AN_{60m}, FV_{60m}, and FS_{60m}. Extractions were carried out using an accelerated solvent extractor (ASE® 200). The total phenol content and ferrous ion binding ability were assessed. The cellular antioxidant activity of the extracts were determined by measuring the activity of antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) and the reduced glutathione (GSH) content in Caco-2 cells incubated with the seaweed extracts for 24 hours. The ability of the extracts to protect against a H_2O_2 and a *tert*-BOOH induced depletion of SOD was also examined. The potential protective effects of the extracts against H_2O_2- and *tert*-BOOH-induced DNA damage were measured by the comet assay. The DNA protective effects of the seaweed extracts were compared against that of the metal chelators deferoxamine mesylate (DFO), 1, 10-phenanthroline (o-phen) and 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (BAPTA-AM).

2. Materials and Methods

2.1. Materials

Human colon adenocarcinoma Caco-2 cells were purchased from the European Collection of Animal Cell cultures (Salisbury, UK). Foetal bovine serum (FBS) was purchased from Invitrogen (Paisley, Scotland). Cell culture plastics were purchased from Cruinn Diagnostics (Greiner Bio-One, Frickenhausen, Germany). Calbiochem SOD Assay Kit II was purchased from Merck Chemicals Ltd. (Nottingham, UK). All other cell culture chemicals and reagents were purchased from Sigma Chemical Co. (Dublin, Ireland). Diatomaceous earth and other materials for generation of ASE® extracts were supplied by DIONEX, (Dionex, UK).

2.2. Preparation of ASE® seaweed extracts

A. nodosum and *F. serratus* were collected from Finnavara, Co. Clare, Ireland. *F. vesiculosus* was harvested from Spiddal, Co. Galway, Ireland during the period of March-April 2010. Seaweeds were freeze-dried at -20˚C for 72 hrs. Freeze-dried macroalgal samples were ground using a pestle and mortar. Extractions were carried out using an Accelerated Solvent Extractor (ASE® 200) equipped with a solvent controller unit. The freeze-dried samples were mixed with silica, at a sample: silica ratio of 1:3 (w/w) and packed into cells containing diatomaceous earth. Prior to solvent extraction an initial heat-up step was employed. Static extractions were performed and the cell was rinsed with the selected extraction solvent (water, ethanol or methanol). The solvent was purged from the cell using N_2 gas and finally depressurization occurred. Between extractions, a cleaning rinse of the complete ASE® system (lines, pump) was carried out using Propan-2-ol to avoid carry-over of extract. The pressure for all extractions was set at 1000 psi, and the temperatures flush volumes and purge times are given in Table 1. All extractions were carried out in triplicate. Extracts were dried using a Labconco® centrifugal vacuum concentrator set at 40 ºC. Dried extracts were stored at -80 ºC until further use. The resulting extracts were termed AN_{100}, AN_{60e}, AN_{80e}, AN_{60m}, FS_{100}, FS_{60e}, FS_{80e}, FS_{60m}, FV_{100}, FV_{60e}, FV_{80e} and FV_{60m}. The samples were reconstituted in 10 ml distilled water to a final concentration of 10 mg/ml.

2.3. Determination of Total Phenol Content (TPC)

The TPC of the extracts was determined as described in O'Sullivan et al. (2011). The absorbance was determined at 765 nm. Gallic acid was used as a standard and TPC was expressed in terms of mg gallic acid equivalents/g dry weight of sample (GAEq/gdw).

2.4. Ferrous ion-chelating ability (FICA) assay

The ferrous ion-chelating ability was determined according to the method of Wang et al. (2009). Briefly, stock solutions of each seaweed extract (5mg/ml) were mixed with distilled water and 2 mM $FeCl_2$. The reaction was initiated by the addition of 5 mM ferrozine. Following 10 mins incubation at room temperature the absorbance was measured at 562 nm. The assay positive control contained distilled water and all assay reagents. The colour blank (containing stock and all reagents apart from ferrozine) was used to correct for colour of the seaweed extracts. The ferrous ion-chelating ability (%) was calculated as follows:

FICA, % = (1 - (Abs_{562nm} Seaweed extract - Abs_{562nm} colour blank) /Abs_{562nm} control$_{))}$ × 100

2.5. Cell culture

Human colon adenocarcinoma Caco-2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with Foetal Bovine Serum (FBS) (10% v/v) and non-essential amino acids (1% v/v). Cells were incubated in an atmosphere of CO_2–air (5:95, v/v) at 37°C and were maintained in the absence of antibiotics. Caco-2 cells were plated at a density of 1×10^5 or 2×10^5 cells/ml for the comet and enzyme assays, respectively.

2.6. Cell viability

Caco-2 cells were supplemented with increasing concentrations (0-2 mg/ml) of seaweed extracts for 24 hrs in 96-well plates with a final volume of 100 µl per well. Cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay (MTT I proliferation kit, Roche Diagnostics; Burgess Hill, West Sussex, UK) as described by O'Sullivan et al. (2011). Sub-toxic concentrations of extracts were determined and used for subsequent experiments.

2.7. Enzymatic and non-enzymatic antioxidant activity

Caco-2 cells were supplemented with seaweed extracts (at concentrations outlined in Table 2) for 24 hrs. Following incubation, the CAT and SOD activities and GSH levels were determined as previously outlined in O'Sullivan et al. (2011). The SOD activity of the cells was also determined following exposure to either 200 μM H_2O_2 or 1 mM *tert*-BOOH for 30 min.

2.8. Determination of DNA damage (Comet assay)

Caco-2 cells were supplemented with seaweed extracts (as outlined in Table 2) for 24 hrs or with 15 mM DFO which chelates iron, 100 μM o-phen which chelates ferrous and copper ions, and 10 mM BAPTA-AM which chelates calcium, for 30 mins in a final volume of 2 ml. The cells were subsequently treated with 50 μM H_2O_2 or 200 μM *tert*-BOOH for 30 mins at 37°C in FBS-free media. DNA damage was measured using the comet assay as previously outlined in O'Sullivan et al. (2011).

2.9. Statistical analysis

Results are presented as mean values ± SE. Statistical analysis was by repeated measures ANOVA followed by Dunnett's test (Prism 4.0, GraphPad Inc, San Diego, CA, USA). The level of statistical significance was $P < 0.05$ or $P < 0.01$.

3. Results and Discussion

3.1. Cell viability

The cytotoxicity of each of the 12 seaweed extracts (3 seaweed extracted using 4 different extraction methods) at concentrations ranging from 0.5 to 2.0 mg/ml was assessed in Caco-2 cells using the MTT assay (Figure 1A). Non toxic concentrations for AN_{100}, AN_{60e}, AN_{60m}, FS_{60e} and FS_{60m} were determined at concentrations between 0.5 and 2.0 mg/ml and the concentrations selected for further investigation are shown in Table 2. The remaining extracts were toxic at concentrations exceeding 0.5 mg/ml therefore the cytotoxicity was also investigated at concentrations ranging from 0.05-0.2 mg/ml (Figure 1B) and sub-toxic concentrations were selected for further investigation (Table 2). Previous studies have related the cytotoxic effects of seaweed extracts to their content of polysaccharides including ascophyllan, fucoidan and phlorotannins (Jiang et al., 2010; Yang et al., 2010).

Extracts of *FV* were the most cytotoxic (Figure 1) and also had the highest total phenol content (Table 3). The total phenol content of *FV* extracts was similar for each of the solvents used in the extraction procedure but differed for the other seaweed species (*FS* and *AN*). The total phenol content of *FS* extracted with 60% MeOH was only 50% to that of the *FS* extracted using water and the total phenol content of *AN* extracts varied from 34.5 mg GAE/gdw (H_2O) to 114.0 mg GAE/gdw (60% EtOH). Cox et al. (2010) found the TPC in methanolic (60%) extracts from a variety of Irish seaweed ranged from 37.6 to 151.3 mg/GAE/g. The polyphenol profile of the samples was not determined but it is known that the polyphenol profiles of different seaweed species may differ significantly (Yoshie et al., 2002) and the extraction process employed will also impact on the concentration and type of polyphenol present in the extract.

Table 1: Conditions of the Accelerated Solvent Extraction (ASE®) procedure.

	Extraction temperature (°C)	Pressure (psi)	Flush volumes	Purge (sec)
100% H_2O	120	1000	2 flush cycles of 60%	90
60% EtOH : 40% H_2O	90	1000	1 flush cycle of 50%	90
80% EtOH : 20% H_2O	100	1000	4 flush cycles of 75%	90
60% MeOH : 40% H_2O	90	1000	1 flush cycle of 50%	90

Table 2: Subtoxic concentrations (mg/ml) of seaweed extracts added to Caco-2 cells following the cell viability assay (MTT).

	H_2O	60% EtOH	80% EtOH	60% MeOH
Ascophyllum nodosum	0.5	0.1	1	1
Fucus serratus	0.1	0.15	0.5	0.5
Fucus vesiculosus	0.1	0.15	0.5	0.2

Figure 1A

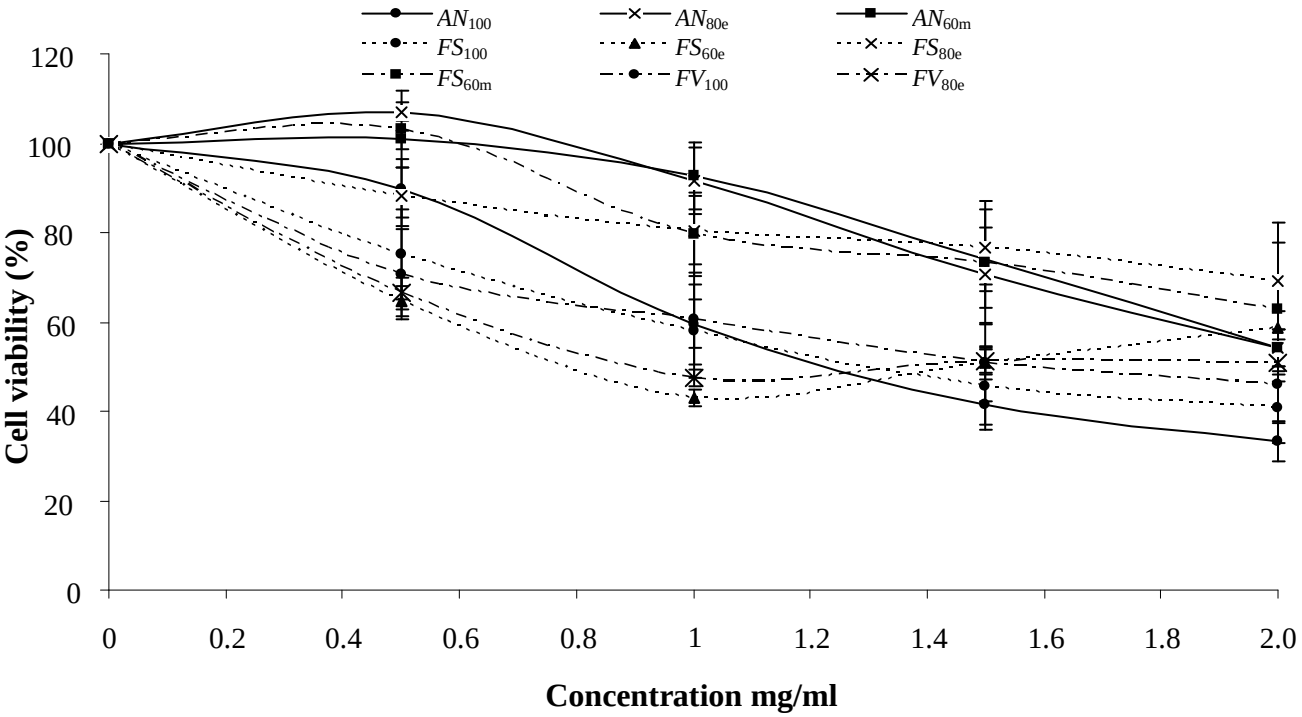


Figure 1B

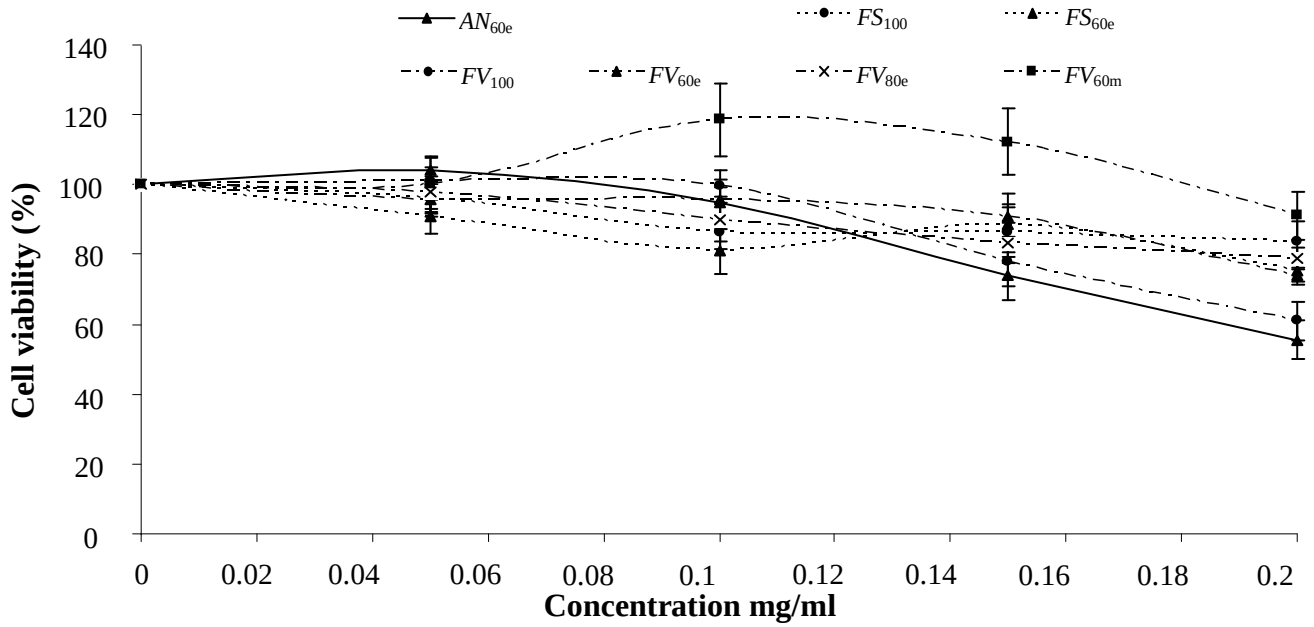


Figure 1A & B: Viability of Caco-2 cells following addition of increasing concentrations (0-2 mg/ml) [**A**] or (0-0.2 mg/ml [**B]** of seaweed extracts as determined by the MTT assay. *A. nodosum* is represented by (—), *F. serratus* is represented by (·····). *F. vesiculosus* is represented by (-··). Solvents are represented as follows: H_2O- ● (AN_{100}, FS_{100}, FV_{100}), 60% EtOH-▲ (AN_{60e}, FS_{60e}, FV_{60e}), 80% EtOH-✕ (AN_{80e}, FS_{80e}, FV_{80e}), 60% MeOH-■ (AN_{60m}, FS_{60m}, FV_{60m}). Data are the means of four independent experiments, with standard errors represented by vertical bars. * Denotes that mean value was significantly different from that of control cells ($P < 0.05$), n=4 independent experiments.

3.2. Enzymatic antioxidant activity

The potential antioxidant effects of the seaweed extracts were examined by measuring the SOD and CAT activities and the GSH content of Caco-2 cells exposed to the seaweed extracts for a 24 hrs period. Previous *in-vivo* studies have shown that supplementation with *Eucheuma cottonii*, (red seaweed) significantly increased SOD activity in hypercholesterolemic rat liver (Wresdiyati et al., 2008). Kim et al. (2008) reported enhanced cellular CAT activity in the erythrocytes of diabetic patients following the consumption of a seaweed supplements consisting of sea tangle (*Laminaria japonica*) and sea mustard (*Undaria pinnatifida*). Previous studies have also demonstrated that seaweeds modulate antioxidant enzymes in cell model systems. The addition of seaweed extracts increased CAT activity in Chinese hamster lung fibroblasts and increased GSH content in Caco-2 cells and HT1080 cells (Kang et al., 2006; O'Sullivan et al., 2011; Kim & Kim, 2010). However, in the present study no enhancement of the antioxidant status was observed following incubation of Caco-2 cells with the seaweed extracts (Figure 2 A, B & C). There was a significant decrease in the reduced GSH content of Caco-2 cells incubated with FV_{60e} (Figure 2C) and in the CAT activity of Caco-2 incubated with the FV_{80e} (Figure 2B). In the previous study the addition of seaweed extracts to Caco-2 cells significantly increased GSH content in the cells. The lack of GSH enhancement in the current study may suggest that the conditions (temperatures of 90-120°C) employed in the ASE® may have degraded heat labile components responsible for the GSH enhancing properties of the extracts used in the previous study (O'Sullivan et al., 2011). The observed reduction in GSH levels by FV_{60e} may be due to presence of quercetin, a flavonoid present in many seaweeds (Yoshie et al., 2003; Namvara et al., 2012). Quercetin has been previously found to reduce GSH content in U937 leukaemia cells (Ramos & Aller, 2008). In addition previous studies have found that a range of polyphenols inhibited glutathione-S-transferase (GST) activity in a number of cell lines (Patra et al., 2008). The observed reduction in CAT activity in Caco-2 cells incubated with FV_{80e} could be an indication of the early stages of a cytotoxic effect. Further study is required to find the mechanisms involved in the reduction of CAT activity and GSH content by certain seaweed extracts.

Methanolic extracts from brown seaweed have demonstrated protective effects against both H_2O_2 and *tert*-BOOH induced reduction in SOD activity (O'Sullivan et al., 2011). Therefore the ability of seaweed extracts to protect against an oxidant-

induced reduction in SOD activity was also examined in the present study. The addition of 200 µM H_2O_2 and 1000 µM *tert*-BOOH to Caco-2 cells reduced SOD activity by approximately 20% compared to the unchallenged control. The extracts did not protect against H_2O_2 or *tert*-BOOH mediated SOD depletion and FV_{60e} exacerbated the H_2O_2-induced decrease in SOD (Figure 3). The extracts employed in this study (produced by ASE®) may not contain the seaweed constituents which previously demonstrated beneficial effects against SOD depletion. High temperature extraction processes have been reported to influence the content and activity of potential antioxidant compounds sourced from plant material (Larrauri et al., 1997).

3.3. Determination of DNA damage

Iron chelation is one of the primary mechanisms in the protection of DNA against oxidant (particularly H_2O_2) induced damage (Barbouti et al., 2001) therefore the ability of each of the seaweed extracts to chelate ferrous iron was assessed. The seaweed extracts extracted using 100% H_2O were the most effective ferrous iron chelators. AN_{100}, AN_{60m} and FS_{100} were found to chelate ferrous ions by 64%, 33% and 43%, respectively (Table 3). The remaining extracts possessed iron chelating capacity at levels which were too low to be accurately quantified. Wang et al. (2009) also found that water derived algal extracts exhibited higher chelation compared to organic solvent (acetone) derived extracts and also found a near inverse relationship between the TPC and the ferrous iron chelation of the extracts which suggests that components other than the polyphenols, such as protein peptides, present in the extracts are responsible for their FICA.

The alkaline single cell gel electrophoresis assay (comet assay) which measures single strand breaks was employed to measure the ability of the seaweed extracts to protect against DNA damage induced by H_2O_2 and *tert*-BOOH. It was found that the addition of 50 µM H_2O_2 to Caco-2 cells increased DNA damage to 55% from a baseline level of 10% (Figure 4A). The addition of 0.5 mg AN_{100} and 1 mg/ml AN_{80e} significantly reduced H_2O_2-induced DNA damage to 35% ($P < 0.01$) and 42% ($P < 0.05$), respectively. However the remaining extracts displayed no protective effects against DNA damage. Previous studies have shown that extracts from seaweeds such as *Grateloupia filicina, Ishige okamurae* and *Ecklonia cava* exhibited protective effects against H_2O_2-induced DNA damage in a range of cellular models (Ahn et al., 2007; Athukorala et al., 2005; Heo & Jeon, 2009).

Brown seaweeds contain phlorotannins, flavonoids and catechins, each of which has previously demonstrated a protective effect against H_2O_2-induced DNA damage (Ahn et al., 2007; Anderson et al., 2001; Kang et al., 2005; Yoshikawa et al., 2004). The protective effects of phlorotannins against H_2O_2-induced DNA damage have been attributed to the catalase stimulatory, GSH stimulatory or hydroxyl scavenging abilities (Ahn et al., 2007; Kang et al., 2005).

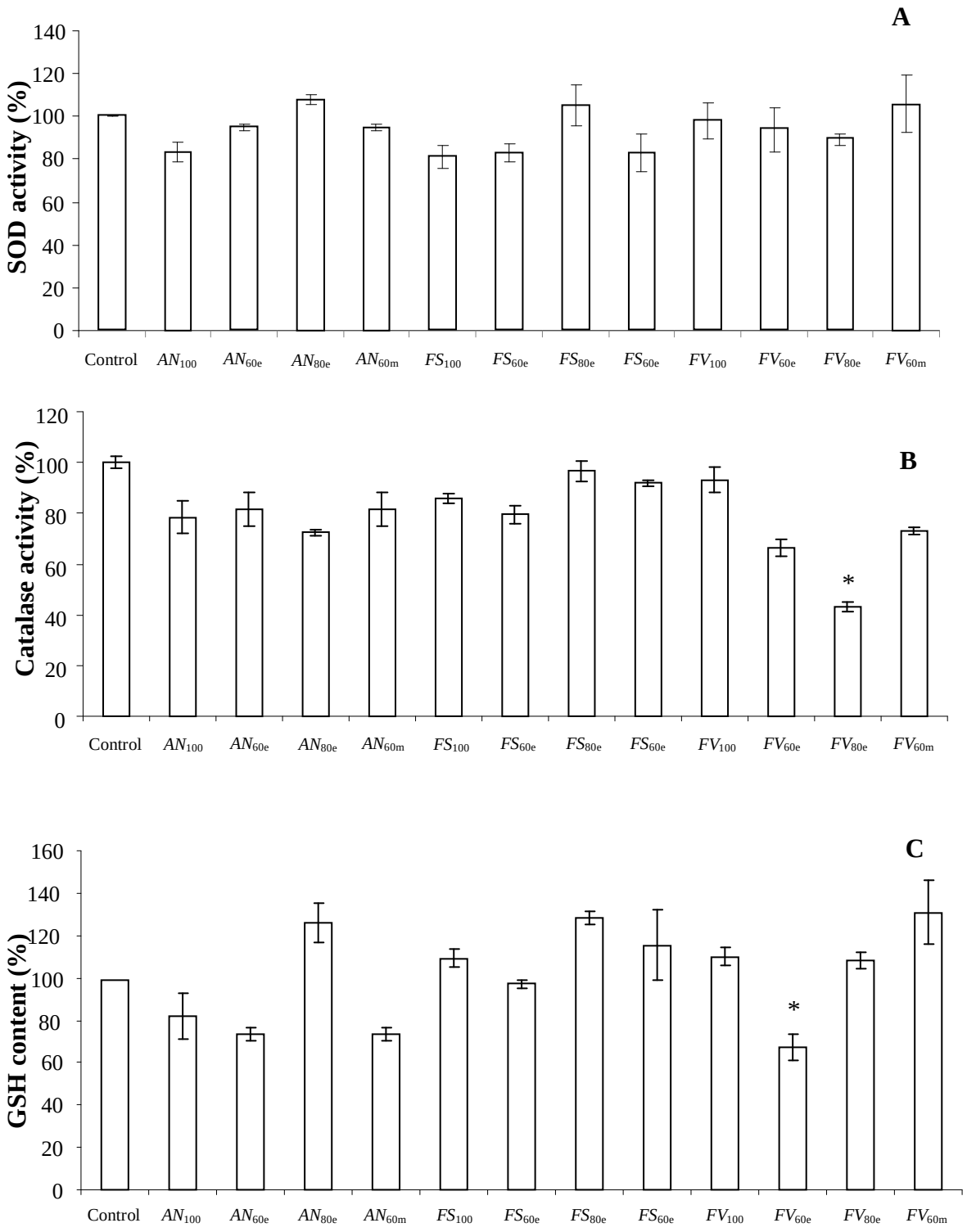


Figure 2: SOD activity (A), catalase activity (B) and GSH content (C) in Caco-2 cells following 24 hrs incubation with seaweed extracts, expressed as a percentage of the control value. Statistical analysis was by repeated measures ANOVA followed by the Dunnett's test. * Denotes that mean value was significantly different from that of control cells ($P < 0.05$), n=4 independent experiments.

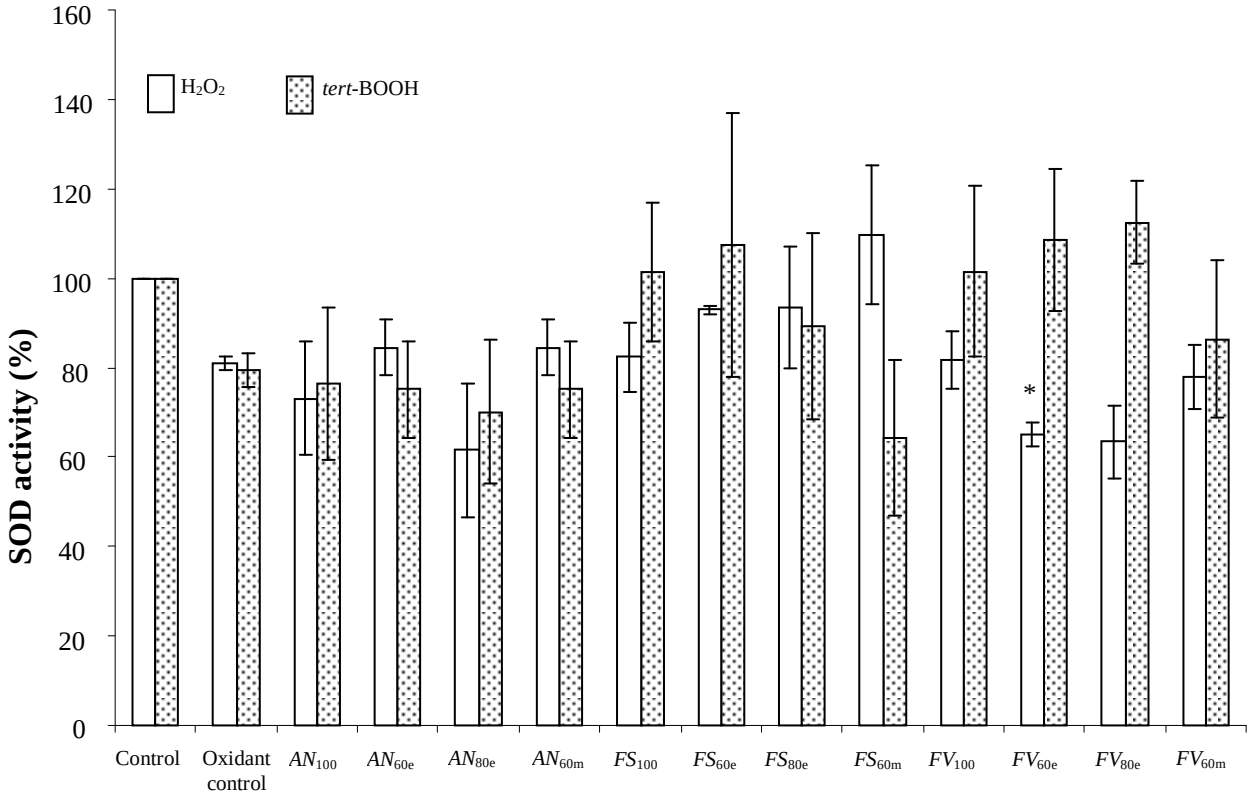


Figure 3: SOD activity in Caco-2 cells following a challenge with H_2O_2 or tert-BOOH in the presence or absence of seaweed extracts. Cells were incubated with seaweed extracts for 24 hrs, media was removed and replaced with media containing 200 μM H_2O_2 or 1000 μM *tert*-BOOH for 30 mins. Statistical analysis was by repeated measures ANOVA followed by the Dunnett's test. * Denotes that mean value was significantly different to that for H_2O_2 or *tert*-BOOH -challenged cells ($P < 0.05$), n=4 independent experiments.

The addition of 200 μM *tert*-BOOH to Caco-2 cells increased DNA damage from a control, untreated level of 10% to 30% (Figure 3B). *tert*-BOOH can induce DNA damage by two mechanisms. Firstly, *tert*-BOOH is metabolised by cytochrome P450 or reacts with iron ions in the Fenton reaction to produce alkoxyl and peroxyl radicals which cause direct DNA damage. A second mechanism involves the depletion of GSH and the oxidation of NADPH causing an imbalance in calcium homeostasis which ultimately leads to cell damage (Ramos et al., 2007). Several of the seaweed extracts in the present study significantly protected against *tert*-BOOH-induced DNA damage, AN_{60e}, AN_{80e}, FS_{100}, FS_{80e} and FV_{60m}, (Figure 4B).

The protective effects of metal chelators DFO, o-phen and BAPTA-AM against both H_2O_2- and *tert*-BOOH-induced DNA damage were compared to those of the seaweed extracts (Figure 4A and B). Metals such as iron and possibly copper may react with H_2O_2 via the fenton reaction to form hydroxyl radicals which cause DNA damage, while the addition of iron chelators such as DFO help prevent DNA damage by binding up excess iron (Barbouti et al., 2001). AN_{100} and AN_{80e} exhibited similar DNA protective effects against H_2O_2-induced damage to that of 15 mM DFO however only AN_{100} chelated ferrous iron (Table 3). This may indicate that AN_{100} protects against DNA damage by binding ferrous iron whilst suggesting that AN_{80e} protects by an alternative mechanism. Phlorotannins and a range of polyphenols commonly found in brown seaweeds like *A. nodosum* have previously been found to protect against H_2O_2-induced DNA damage by binding free iron (Lüder & Clayton, 2004; Cox et al., 2010). However, the present study found that extracts with the highest polyphenol content neither chelated ferrous iron nor protected against H_2O_2-induced DNA damage. Several of the extracts exhibited similar levels of protection against *tert*-BOOH-induced DNA damage to that of the three chelators. Of these extracts FS_{100} exhibited a strong ferrous ion chelating ability indicating that this extract may have reduced DNA damage by binding up ferrous ions. The absence of ferrous iron chelation in the remaining extracts may suggest that these extracts contain alternative components which help protect DNA against the more complex mechanisms involved in *tert*-BOOH induced damage (Ramos et al., 2007).

To summarise, the seaweed extracts did not influence the CAT and SOD activity or GSH content of unchallenged Caco-2 cells, or protect against H_2O_2 or *tert*-BOOH-induced SOD depletion in Caco-2 cells. A number of seaweed extracts protected

against H_2O_2 and *tert*-BOOH-induced DNA damage in Caco-2 cells, however the nature of their protective mechanisms could not be fully established.

Table 3: Total phenol content (TPC) (mg GAE/gdw) and ferrous ion-chelating ability (FICA) (%) of the seaweed extracts.

	H_2O (1)		60% EtOH (2)		80% EtOH (3)		60% MeOH (4)	
	TPC	FICA	TPC	FICA	TPC	FICA	TPC	FICA
AN (a)	(a2,c1,c2,c3,c4) 34.5±5.5	(a4,b2) 64.0±1.5	(a1,a3,a4,b1,b2,b3,b4) 114.0±5.5	NQ	(a2,c1,c2,c3,c4) 50.2±12.5	NQ	(a2,c1,c2,c3,c4) 55.5±25.5	(a1) 33.0±1.0
FS (b)	(a2,c1,c2,c3,c4) 40.7±1.0	(a1) 43.0±7.5	(a2,c1,c2,c3,c4) 20.5±2.5	NQ	(a2,c1,c2,c3,c4) 28.0±0.5	NQ	(a2,c1,c2,c3,c4) 19.7±7.5	NQ
FV (c)	(a1,a3,a4,b1,b2,b3,b4) 110.7±9.0	NQ	(a1,a3,a4,b1,b2,b3,b4) 104.0±19.5	NQ	(a1,a3,a4,b1,b2,b3,b4) 99.5±5.5	NQ	(a1,a3,a4,b1,b2,b3,b4) 94.6±6.5	NQ

(a1–c4) Denote a significant difference between mean values, where a1 denotes a significant difference from AN_{100} and a2 denotes a significant difference from AN_{60e}, etc. NQ = not quantifiable. Repeated measures ANOVA followed by Tukey's post-test, n = 4 independent experiments. Mean ± SE.

Figure 4

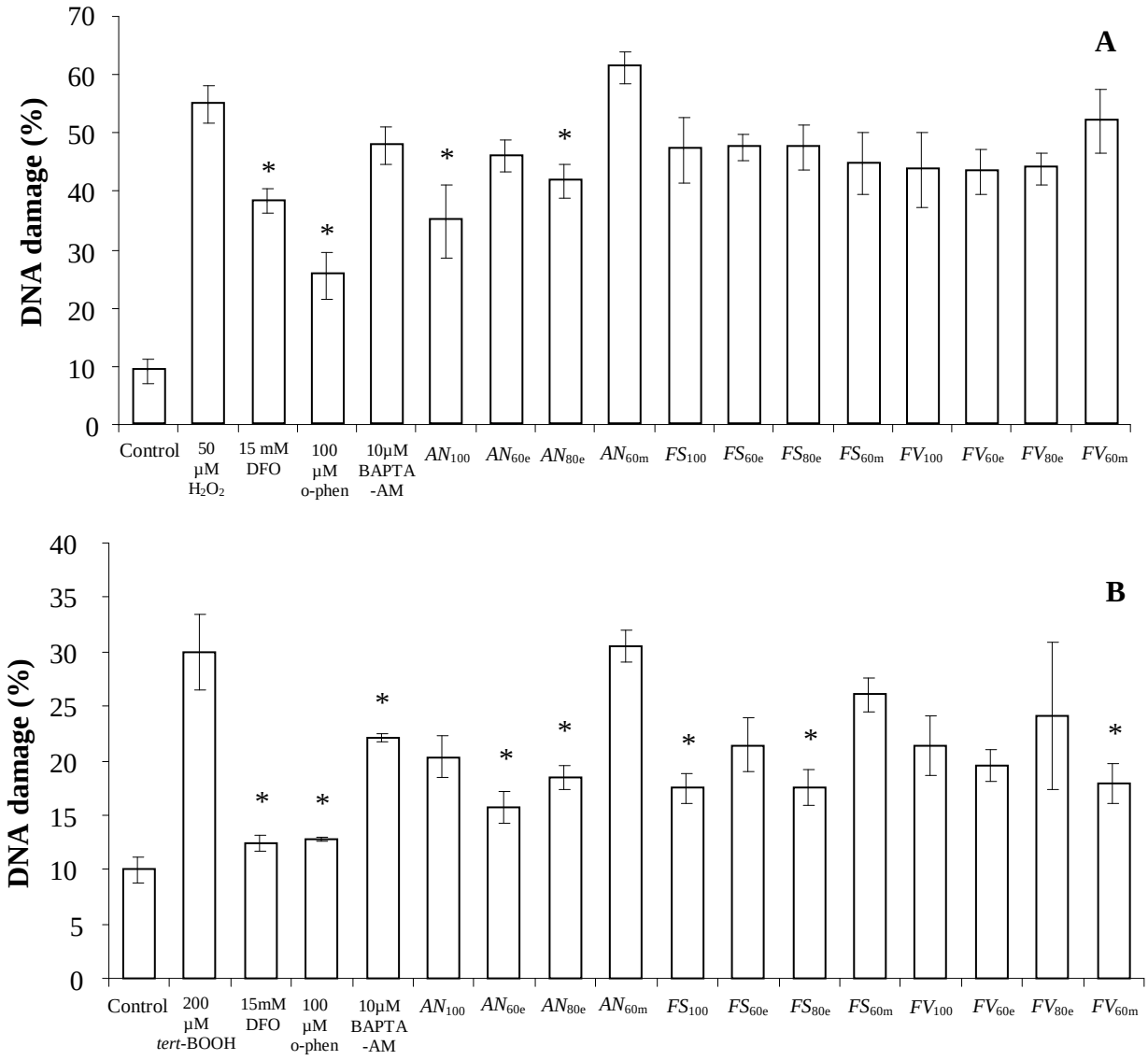


Figure 4: DNA damage in Caco-2 cells, expressed as percent tail DNA. Cells were incubated with metal chelators, 15 mM desferoxamine mesylate, 100 µM 1,10-phenanthroline, 10 µM BAPTA-AM for 30 mins or seaweed extracts for 24 hrs, media was removed and replaced with media containing 50 µM H_2O_2 [A] or 200 µM *tert*-BOOH [B] for a further 30 mins. Statistical analysis was by repeated measures ANOVA followed by the Dunnett's test. * Denotes that mean value was significantly different from that for H_2O_2 or *tert*-BOOH -challenged cells ($P < 0.05$), n=4 independent experiments.

4. Conclusions

None of the solvent ratios employed in the current study produced extracts which exhibited positive antioxidant properties in all of the cell culture based assays however, *A. nodosum* extracts were seen to be the most effective at protecting against H_2O_2 and *tert*-BOOH-induced DNA damage. Overall, of the three seaweeds investigated, *A. nodosum* displayed the most potential as a source of antioxidant compounds.

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CHAPTER 5

An examination of the potential of seaweed extracts as functional ingredients in milk.

Abstract

Extracts were manufactured from *Ascophyllum nodosum* with 100% water (AN_{100}) and 80% ethanol (AN_{80e}) and *Fucus vesiculosus* with 60% ethanol (FV_{60e}) using solid-liquid extraction techniques. Milk containing seaweed extracts (AN_{100}, AN_{80e} and FV_{60e}) at 0.25 and 0.5% (w/w) were prepared and stored for up to 11 days at 4°C. Phloroglucinol (Phl) (0.5% w/w) was run as a positive control. Quality and shelf-life parameters, stability and bioactivity of seaweed extracts in milk were examined over the 11 day storage period. Milk samples containing AN_{80e} (0.25%), AN_{80e} (0.5%), FV_{60e} (0.25%) and FV_{60e} (0.5%) had significantly ($P < 0.05$) higher "-a*" greenness and "b*" yellowness values than the other milk samples. On day 11 of storage, milk samples containing FV_{60e} (0.25%) and Phl (0.5%) had significantly ($P < 0.05$) lower lipid oxidation. pH and microbiology (total plate count, coliforms, yeasts and moulds) of milk was not affected by seaweed type or concentration. Sensory analysis indicated the control and AN_{100} (0.5%)-enriched milk samples were the most acceptable by panellists. Milk containing AN_{80e} (0.5%) and FV_{60e} (0.25%) were significantly positively ($P < 0.05$) correlated with a fishy taste and consequently were unacceptable to sensory panellists.

Seaweed extracts in milk were stable (DPPH radical scavenging) as a function of storage time. Milk and milk digestates exhibited DPPH radical scavenging and ferrous ion chelating (FICA) activities. Milk and milk digestates did not affect the antioxidant status (CAT, SOD and GSH activity) or protect against H_2O_2-induced DNA damage in Caco-2 cells. Results indicate that seaweed extracts have limited potential to enhance fresh milk quality. Seaweed extracts did not display bioactivity when added to milk as functional ingredients.

1. Introduction

Milk is a highly important food in many countries due to its nutritional properties and use in the manufacture of a wide variety of dairy products such as milk powder, yogurt, and cheese. In recent years a range of milk products enriched with functional ingredients have been manufactured. Functional foods are defined as food components which provide demonstrated physiological benefits or reduce the risk of chronic disease beyond their basic nutritional function (Shah, 2001). Dairy-based functional food products account for approximately 43% of the total market share however the majority of products are fermented dairy foods (Özer & Kirmaci, 2009). In Ireland, the most prominent example of a functional milk product is Avonmore Super Milk® (Glanbia Dairies, Kilkenny) which is enriched with folic acid, calcium, protein and a number of vitamins. A number of fibre, omega-3 fatty acid, immunoglobulin, melatonin, and phytosterol-enriched milk products are also commercially available (Saxelin et al., 2003). The scientific literature reports a number of studies where milk is examined as a potential carrier for a range of functional ingredients. The majority of studies are based on the manufacture of milk enriched with fatty acids from fish (Let at al., 2004; Mette et al., 2007), algal oil (Gallaher et al., 2005) and vegetable oil sources (Gutiérrez et al., 2009). Other studies have examined phytosterol (Menéndez-Carreño et al., 2008; Noakes et al., 2005), and fibre (Lópezn et al., 2008) enriched milk, as well as lactose-free milk (Lummela et al., 2009).

Antioxidants have been linked to a range of health benefits including a reduced incidence of cancer, heart disease, diabetes and many other diseases (Kaur & Kapoor, 2001). A limited number of studies have investigated milk as a potential carrier for antioxidant compounds with the aim of increasing the health benefits and quality attributes of the resulting product. Gad & Salam (2010) reported that the addition of rosemary or green tea extracts increased the antioxidant activity (total phenol content (TPC), DPPH radical scavenging and ferric reducing antioxidant power (FRAP) activities) of the enriched milk. Similarly, the addition of apple polyphenol enhanced the antioxidant activity (TPC, oxygen radical absorbance capacity (ORAC), and FRAP activity) of milk (Wegrzyn et al., 2008). Bilberry and black currant extracts were found to significantly increase the ORAC and DPPH-scavenging activity of the resulting milk (Skrede et al., 2004). Axten et al. (2008)

manufactured UHT milk containing extracts from grape seed or apple, however the effects of supplementation were only examined from a sensory perspective. It was concluded that a high level of bitterness rendered apple extracts unsuitable ingredients for incorporation into milk.

Seaweed represents an alternative source of antioxidant compounds (Budhiyanti et al., 2011). In previous studies carried out in our research group extracts from *Ascophyllum nodosum* (100% H_2O (AN_{100}) and 80% ethanol (AN_{80e}) extracts) and *Fucus vesiculosus* (60% ethanol extract (FV_{60e})) exhibited a range of *in-vitro* and cellular antioxidant activities (Chapter 4). The objective of this study was to manufacture milk enriched with AN_{100}, AN_{80e} and FV_{60e}. The effect of seaweed extracts (0.25 and 0.5%) (w/w) on the quality and shelf-life (colour, lipid oxidation, pH, and microbiology) and sensory characteristics of milk was investigated over an 11 day storage period. Phloroglucinol (Phl) (0.5% w/w), the monomeric building unit of phlorotannins (phenolic compounds present in brown algae) was run as a positive antioxidant control. The stability of seaweed extracts in milk was assessed using the DPPH radical scavenging activity assay. Seaweed extract enriched milk was subjected to an *in-vitro* digestion procedure to assess the antioxidant capacity of the milk before and after digestion. Antioxidant activity of the milk and milk digestates was determined using *in-vitro* antioxidant (DPPH radical scavenging activity and ferrous iron-chelating activity (FICA)) and cellular antioxidant (Catalase (CAT), superoxide dismutases (SOD) and glutathione (GSH)) assays. The ability of milk and milk digestates to protect against oxidant-induced DNA damage in human adenocarcinoma Caco-2 cells was also investigated.

2. Materials and methods

2.1. Materials

Fresh milk was obtained from a local farm in Cork. Human colon adenocarcinoma Caco-2 cells were purchased from the European Collection of Animal Cell Cultures (Salisbury, UK). Foetal bovine serum (FBS) was purchased from Invitrogen (Paisley, Scotland). Cell culture plastics were purchased from Cruinn Diagnostics (Greiner Bio-One, Frickenhausen, Germany). Calbiochem superoxide dismutases (SOD) Assay Kit II and Catalase (CAT) kit was purchased from Merck Chemicals Ltd. (Nottingham, UK). Agar was purchased from Oxoid Ltd. (Basingstoke, Hampshire, England) and Merck Chemicals Ltd. (Cork, Ireland). All other cell culture chemicals and reagents were purchased from Sigma-Aldrich Ireland Ltd., Arklow, Co. Wicklow, Ireland.

2.2. Preparation and characterisation of seaweed extracts

Ascophyllum nodosum was collected from Finnavara, Co. Clare, Ireland. *Fucus vesiculosus* was harvested from Spiddal, Co. Galway, Ireland. Seaweeds were freeze-dried at -20˚C for 72 hrs, vacuum-packed and stored at -80ºC prior to extraction. Seaweed extracts were manufactured from freeze-dried seaweed using a simple solid-liquid extraction and one of three solvent systems at room temperature: water (100%) and ethanol/water (80 : 20) for *Ascophyllum nodosum* (AN_{100} and AN_{80e}) and ethanol/water (60 : 40) for *Fucus vesiculosus* (FV_{60e}).

Dried seaweed (250 g) was ground to a fine powder using a blender (Waring Laboratory Science, USA). In water (100%) and ethanol-based extractions (80 : 20 or 60 : 40 ethanol/water) ground seaweed was suspended in the extracting solvent at ratios of 1 : 20 and 1 : 10 (w/v), respectively. Water and ethanol extractions were carried out by incubation in an orbital shaker (MaxQ 6000 Shaker, Thermo Fisher Scientific, Ireland) at 175 rpm. Water extracts were filtered after 6 hrs incubation, the seaweed material was re-suspended in water (1 : 20 (w/v)) and filtered after 24 hrs through glass wool filters due to the viscosity of the resulting extract. The water extracts were combined and concentrated by rotary evaporation (Buchi Rotavapor R-220, Mason Technologies, Ireland) at 50ºC.

Ethanol extracts were filtered through a Buchner funnel after 3 hrs incubation and the seaweed material was re-suspended in ethanol/water (1 : 10 (w/v)) for an

additional 3 hrs. This process was repeated with a final overnight extraction to ensure exhaustive extraction occurred. The solvent extracts were combined and ethanol was removed by rotary evaporation at 50°C. All seaweed extracts (AN_{100}, AN_{80e} and FV_{60e}) were subsequently freeze-dried, ground and stored at -80ºC prior to analysis The antioxidant activity of AN_{100}, AN_{80e} and FV_{60e} and phloroglucinol (Phl) was determined using *in-vitro* antioxidant assays (total phenol content (TPC), DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate) radical scavenging activity and ferrous-ion chelating activity (FICA)) assays.

2.3 *In-vitro* antioxidant assays

2.3.1. Total phenol content (TPC)

The TPC of the seaweed extracts and phloroglucinol was measured according to the method of Singleton & Rossi (1965). Gallic acid was used as a standard and TPC was expressed in terms of mg gallic acid equivalents/g dry weight (GAE/g).

2.3.2. DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate) radical scavenging activity

Seaweed extracts and Phl were dissolved in methanol at a concentration of 1 mg/ml. Seaweed extracts and Phl (500 µl) were added to 3.5 ml DPPH solution (in methanol) (100 µM) and mixed vigorously using a vortex mixer. Samples were stored in the dark for 60 min and subsequently centrifuged (Sigma 4K15, Sigma Centrifuge GmbH, Osterode am Harz, Germany) at 3000g for 10 min at room temperature. The absorbance of the supernatant was measured at 515 nm using a spectrophotometer (WPA lightwave, Biochrom, Cambridge, UK). Trolox (0.04 µM) was used as a standard for comparative purposes. A control was prepared containing DPPH and methanol only. Data was expressed as % DPPH radical scavenging activity and calculated as follows:

$$\text{DPPH activity, \%} = (1 - (\text{Abs}_{515nm}\ \text{Seaweed extract} / \text{Abs}_{515nm}\ \text{Control})) \times 100$$

2.3.3. Ferrous-ion chelating activity

Ferrous-ion chelating activity **(**FICA) was carried out as described by Wang et al. (2009). In a 96-well plate 100 µl of each seaweed extract (1 mg/ml) and Phl (1 mg/ml) were mixed with 150 µl distilled water and 5 µl $FeCl_2$ (2 mM). The reaction was initiated by the addition of 5 µl ferrozine (5 mM). Following 10 mins incubation at room temperature the absorbance was measured at 562 nm using a

plate reader (Thermo Scientific Varioskan Plate Reader, Fisher Scientific UK Ltd). The assay positive control contained distilled water and all assay reagents. The colour blank (containing seaweed extracts and all reagents apart from ferrozine) was used to correct the colour of the seaweed extracts. The % ferrous ion-chelating activity (FICA) was calculated as follows:

FICA, % = (1 - ((Abs_{562nm} Seaweed extract - Abs_{562nm} Colour blank) /Abs_{562nm} Control)) × 100

2.4. Preparation of seaweed extract enriched milk

Fresh milk was homogenised (Model APV 1000, APV homogenisers AS, Albertslund, Denmark) using a two-step process (150 bar (1st stage) and 30 bar (2nd stage)). Seaweed extracts (AN_{100}, AN_{80e}, and FV_{60e}) were added to milk at concentrations of 0.25% and 0.5% (w/w). Phl (0.5% w/w) was added to milk as a positive antioxidant control. Milk samples were subsequently stirred for 5 hrs at 4°C with the aid of a magnetic stirrer to aid dissolution. Milk containing seaweed extracts and Phl was batch pasteurised in a waterbath at 63°C for 30 minutes, and subsequently cooled. The resulting milk sample were termed AN_{100} (0.25%), AN_{100} (0.5%), AN_{80e} (0.25%), AN_{80e} (0.5%), FV_{60e} (0.25%), FV_{60e} (0.5%) and Phl (0.5%). Milk (240 ml) was aseptically packaged in 250 ml polypropylene containers (Sarstedt Ltd., Co. Wexford, Ireland) and stored for 11 days at 4°C.

2.5. Surface colour measurement of milk

The surface colour of milk samples was measured using a Minolta CR-400 colorimeter (Minolta Camera, Co. Ltd., Japan). The chroma meter consisted of a measuring head (CR-400), with an 8 mm diameter measuring area, a 2° standard observer, and a data processor (DP-400). The chroma meter was calibrated using the CIE Lab colour space system using a white tile (C: Y = 93.6, x = 0.3130, y = 0.3193) and Yxy values were converted to L*a*b*. The 'L*' value represents lightness and '-a*' and 'b*' values represent greenness and yellowness, respectively. Six measurements were recorded and an average value was reported per milk sample. Colour measurements of the milk were recorded on days 1, 4, 6, 8 and 11 of storage.

2.6. Measurement of lipid oxidation in milk

Lipid oxidation in milk was measured following the procedure of King et al. (1962) with modifications. Milk (20 ml), 100% TCA (1.15 ml) and 95% ethanol (2.3 ml) were added to a 50 ml screw capped plastic tube (Sarstedt Ltd., Wexford, Ireland) and the contents were shaken vigorously. The mixture was filtered through Whatman No. 42 filter paper. The resulting filtrate was added to 0.1M TBA reagent (dissolved in 95% ethanol) in screw cap test tubes, placed in a water bath (Model B21, Fisher Scientific Ltd., Dublin, Ireland) at 60°C for 1 hr and subsequently filtered through Whatman No. 1 filter paper. Following cooling, the absorbance of the TBA-malondialdehyde complex was measured on a spectrophotometer (Cary 300 Bio, UV-Vis spectrophotometer, Varian Instruments, CA, USA) at 532 nm against a blank containing all assay reagents. The malondialdehyde content of yoghurt was calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. Results were expressed as 2-thiobarbituric acid-reactive substances (TBARS) in mg malondialdehyde (MDA)/kg milk. Lipid oxidation in milk was measured on days 1, 4, 6, 8 and 11 of storage.

2.7. pH measurement of milk

The pH of the milk was measured using a pH meter (Seven Easy portable, Mettler-Toledo GmbH, Switzerland) by directly inserting the pH probe into the milk samples. The pH of the milk was recorded on days 1, 4, 6, 8 and 11 of storage.

2.8. Microbiological analysis of milk

Milk (10 ml) was transferred into stomacher bags, diluted with 90 ml of Ringer's solution and stomached for 1 min (Steward Stomacher 400 Lab Blender, London, UK) resulting in a 1/10 dilution used for analysis. Serial dilutions were prepared and colony counts were determined using the pour plate technique with plate count agar (PCA), Violet Red Bile (VRB) Agar (Oxoid Ltd.) and Yeast Extract Glucose Chloramphenicol (YGC) Agar (Merck Millipore, Cork, Ireland) which assesses total plate count, total coliforms, yeasts and moulds, respectively. The plates were incubated at 30°C for 3 days and results were expressed as $\log_{10}$CFU (colony forming units)/ml milk. Microbiological analysis of milk samples was carried out on days 1, 4, 6, 8 and 11 of storage.

2.9. Sensory evaluation

Sensory analysis was performed using 26 naive assessors (20 – 40 years age range) on days 1, 4, 8 and 11 of storage. Sensory analysis descriptors were colour, fishy taste, odour, flavour, off-flavour, texture and overall acceptability. Assessors were asked to indicate their degree of liking on a 10 cm line scale ranging from 0 (extremely dislike) to 10 (extremely like).

Sensory analysis was undertaken in the panel booths of the UCC sensory laboratory which conforms to ISO (1988) international standard. Seven coded samples were given to each panellist and they were required to rinse their mouths with water before tasting each sample. The order in which the samples were presented was randomised to prevent potential flavour carryover effects (MacFie et al., 1989).

2.10. Stability and bioactivity of seaweed extract enriched milk

2.10.1. Stability of seaweed extracts in milk

The stability of seaweed extracts in milk samples over the storage period was determined using the DPPH radical scavenging activity assay as described in section 2.3.2. Milk samples were diluted in methanol (1:10) and 500 µl was added to 3.5 ml DPPH stock (100 µM) and measured as described above. The % DPPH radical scavenging activity (stability) of milk was measured on days 1, 4, 6, 8 and 11 of storage.

2.10.2. *In-vitro* digestion of milk

In-vitro digestion of milk samples was carried out following the method of Daly et al. (2010) with modifications. All experimental work was carried out in UV-light free conditions to reduce the possible photo-decomposition of the antioxidant extracts present in the milk. Milk samples (containing 0.5% AN_{100}, AN_{80e}, FV_{60e} or Phl)) (1 g) were dissolved in 10 ml HBSS and shaken vigorously. Aliquots (5 ml) were transferred to amber bottles. To mimic the gastric phase of digestion, 100 µl pepsin (0.04 g/ml in 0.1 N HCl) was added to each bottle and the pH was adjusted to 2 using 1 N HCl. Oxygen was removed by blowing nitrogen over the samples. Samples were incubated at 37ºC for 1 hr in an orbital shaking (95 rpm) water bath (Grant OLS200, Keison Products, Essex, UK). Following gastric digestion, the pH was increased to 5.3 using sodium carbonate (0.9 M $NaHCO_3$) followed by the addition of 200 µl bile salts (1.2 mg/ml glycodeoxycholate, 0.8 mg/ml taurocholate

and 1.2 mg/ml taurodeoxycholate) and 100 µl pancreatin (0.08 g/ml HBSS). The pH was subsequently adjusted to 7.4 using NaOH, oxygen was displaced by nitrogen and samples were incubated at 37°C in the orbital shaking water bath for a further 2 hrs. Following simulated intestinal digestion, the digested milk samples were ultracentrifuged (Beckman L7–65 ultracentrifuge; Palo Alto, CA, USA) at 223,487 g for 95 min to isolate the micelle (aqueous) fraction. Digestate samples were filter sterilised using 0.22 µm syringe-driven filters (Merck-Millipore, Cork).

2.10.3. Effect of digestion on the antioxidant stability of seaweed enriched milk

Milk and subsequent digestions were diluted in water or methanol (1:10) for FICA and DPPH radical scavenging activity, respectively (section 2.3).

2.10.4. Cellular antioxidant activity of milk and milk digestates

Human colon adenocarcinoma Caco-2 cells were maintained as described in O'Sullivan et al. (2011). Caco-2 cells were supplemented with increasing concentrations (0-10% v/v) of undigested and digested milk samples for 24 hrs. Cell viability was assessed using the neutral red assay as described by Aherne et al. (2007). Sub-toxic concentrations, at which cell viability of Caco-2 cells was unaffected, of seaweed extract enriched milk and milk digestates were determined and used for subsequent experiments (Table 7). The Comet assay was used to access the potential DNA protective effects of the seaweed extract and Phl enriched milk and milk digestates in Caco-2 cells following 30 min treatment with 50 µM H_2O_2. For the determination of cellular enzymatic activity, Caco-2 cells were supplemented with seaweed enriched milk and milk digestates (at concentrations outlined in Table 8) for 24 hrs. Following incubation, the CAT and SOD activities and GSH levels were determined.

2.11. Statistical Analysis

Statistical analysis for *in-vitro* antioxidant assays, surface colour, lipid oxidation, pH, and microbiology measurements was by one-way ANOVA or repeated measures ANOVA followed by Dunnett's test or Tukey's test (Prism 4.0, GraphPad Inc, San Diego, CA, USA). Each experiment was carried out three times and results are presented as mean values ± the standard error of the mean (SEM). The level of statistical significance was $P < 0.05$.

ANOVA-Partial Least Squares Regression (APLSR) was used to process the mean data accumulated from the 26 test subjects during sensory analysis and shelf-life evaluation using instrumental methods. The X-matrix was designated as 0/1 for treatment and days with the Y-matrix designated as sensory and instrumental variables. The optimal number of components in the ASLSR models presented was determined to be 6 principal components. Principal component analysis (PCA) is a mathematical technique that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables called principal components. PC1 versus PC2 is presented as the other PCs did not yield additional information. In these models, assessor and session level effects were removed using level correction. The validated explained variance for the model constructed for milk samples was 44.19% and the calibrated variance was 44.19%. To derive significance indications for the relationships determined in the quantitative APLSR, regression coefficients were analyzed by jack-knifing which is based on cross-validation and stability plots (Martens and Martens, 1999, 2001). Jack-knifing is a method used in statistical inference to estimate the bias and standard error (variance) of a statistic, when a random sample of observations is used to calculate it. All analyses were performed using Unscrambler Software, version 9.8 (CAMO ASA, Norway).

3. Results and Discussion

3.1. Characterisation of seaweed extracts

The total phenol content of the seaweed extracts followed the order Phl > FV_{60e} > AN_{80e} > AN_{100} (Table 1) and the TPC of these extracts were comparable to those of the ASE® extracts from Chapter 4. By contrast the FICA of the FV_{60e} and Phl were significantly lower ($P < 0.05$) than that of AN_{100} and AN_{80e} . These results highlight the complexity and range of compounds present in seaweed extracts which exhibit a variety of chemical activities. The DPPH radical scavenging activity was similar for all seaweed extracts and Phl results reported were comparable to the DPPH radical scavenging activity of 60% methanol prepared seaweed extracts reported in Chapter 2. Prior to the production of seaweed extract-enriched milk the maximum solubility of the seaweed extracts was tested at concentrations ranging from 0.25% to 5% (w/w). Each of the seaweed extracts was fully soluble in milk at concentrations of 0.25% and 0.5% (w/w), however their solubility rapidly decreased at concentrations greater than 0.5% (w/w). Seaweed extracts were added to milk at concentrations of 0.25% and 0.5% (w/w) for the remainder of the trial.

3.2. Surface colour measurement of milk

The seaweed extracts used in the present study may contain a range of pigments such carotenoids, chlorophyll and polyphenols (Schoefs, 2004) which may influence the colour of the seaweed extract enriched milk (Sanz et al., 2008). On day 1 of storage, milk containing FV_{60e} (0.5%) and AN_{80e} (0.5%) had significantly lower ($P < 0.05$) "L*" values than all other milk samples. Over time the "L*" values of most milk samples decreased. On day 11 of storage, milk containing FV_{60e} (0.5%) ($P < 0.05$) and AN_{80e} (0.5%) ($P < 0.05$) had lower "L*" values compared the control however their values were not significantly different from the other milk samples. Milk containing AN_{80e} (0.25%), AN_{80e} (0.5%), FV_{60e} (0.25%), and FV_{60e} (0.5%) had significantly higher "-a*" greenness values compared to the other milk samples examined. The increase in '-a*' greenness values may be due to trace amounts of chlorophyll present in the seaweed extracts. Milk samples containing AN_{80e} (0.25%), AN_{80e} (0.5%), FV_{60e} (0.25%), and FV_{60e} (0.5%) had significantly higher ($P < 0.05$) "b*" yellowness values compared to the other milk samples which may be due to the presence of yellow pigments such as fucoxanthin, rutin or morin in the seaweed

extracts which can be extracted using ethanol (Strain et al., 1944; Yoshie-Stark et al., 2003).

Table 1

Total phenol content (TPC), 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH•) radical scavenging assay and ferrous-ion chelating assay (FICA) of seaweed extracts and phloroglucinol.

	AN_{100}[a]	AN_{80e}[b]	FV_{60e}[c]	Phl[d]
TPC (mg gallic acid equivalent)	43.5 ± 5.5[b,c,d]	74.8 ± 3.5[a,c,d]	91.5 ± 6.5[a,b]	106.5±2.0[a,b]
DPPH (% radical scavenging)	28.0 ± 3.0	26.5 ± 4.0	28.5 ± 5.0	30.5±1.5
FICA (%)	68.0 ± 8.0[c,d]	75.5 ± 7.5[c,d]	44.5 ± 6.0[a,b]	26.5±6.5[a,b,c]

([a-d]) denotes a significant difference between mean values, repeated measures ANOVA followed by Tukey's post-test. Data without superscripts indicates no significant difference n= 4 independent experiments.

Table 2

Effect of seaweed extract addition on the surface lightness ("L*" value), greenness ("-a*") and yellowness ("b" value) of milk at 11 days at 4°C.

		Storage time at 4°C,days				
		1	4	6	8	11
Lightness "L*"	Control[a]	88.9±1.3[e,g]	89.7±0.4[e,g]	89.4±1.5[e,g]	86.1±1.9[e,g]	86.3±1.0[e,g]
	AN_{100} (0.25%)[b]	87.7±1.9[e,g]	88.6±0.4[e,g]	85.1±1.4[e,g]	85.2±1.3[e,g]	84.5±1.0[e,g]
	AN_{100} (0.5%)[c]	84.6±1.0[e,g]	86.9±1.0[e,g]	84.2±1.2[e,g]	83.8±0.9[e,g]	83.7±1.6[e,g]
	AN_{80e} (0.25%)[d]	83.5±2.8[e,g]	85.2±1.6[e,g]	82.0±2.3[e,g]	81.1±1.4[e,g]	83.7±0.8[e,g]
	AN_{80e} (0.5%)[e]	81.4±2.1[a-d,f,h]	80.9±1.9[a-d,f,h]	83.7±1.3[a-d,f,h]	82.8±1.6[a-d,f,h]	82.7±1.0[a-d,f,h]
	FV_{60e} (0.25%)[f]	83.5±0.9[e,g]	84.1±0.7[e,g]	84.4±1.4[e,g]	85.3±1.7[e,g]	85.1±1.1[e,g]
	FV_{60e} (0.5%)[g]	81.2±1.5[a-d,f,h]	80.8±2.1[a-d,f,h]	81.8±1.3[a-d,f,h]	83.4±1.6[a-d,f,h]	84.2±2.1[a-d,f,h]
	Phl (0.5%)[h]	87.7±1.5[e,g]	88.2±2.3[e,g]	86.1±2.4[e,g]	87.7±1.9[e,g]	87.5±2.4[e,g]
Greenness "-a*"	Control[i]	-5.1±0.3[l-o]	-5.0±0.2[l-o]	-5.1±0.2[l-o]	-4.8±0.3[l-o]	-4.9±0.2[l-o]
	AN_{100} (0.25%)[j]	-4.7±0.4[l-o]	-4.4±0.2[l-o]	-5.1±0.3[l-o]	-5.7±0.2[l-o]	-5.9±0.3[l-o]
	AN_{100} (0.5%)[k]	-4.3±0.3[l-o]	-3.9±0.3[l-o]	-4.8±0.3[l-o]	-5.8±0.2[l-o]	-5.8±0.3[l-o]
	AN_{80e} (0.25%)[l]	-6.8±0.6[i-k,p]	-6.4±0.5[i-k,p]	-6.7±0.5[i-k,p]	-6.8±0.5[i-k,p]	-6.6±0.5[i-k,p]
	AN_{80e} (0.5%)[m]	-7.2±0.5[i-k,p]	-6.9±0.4[i-k,p]	-6.1±0.4[i-k,p]	-6.2±0.5[i-k,p]	-6.2±0.5[i-k,p]
	FV_{60e} (0.25%)[n]	-7.4±0.5[i-k,p]	-6.6±0.4[i-k,p]	-6.7±0.4[i-k,p]	-4.9±0.5[i-k,p]	-4.9±0.4[i-k,p]
	FV_{60e} (0.5%)[o]	-7.2±0.4[i-k,p]	-7.0±0.4[i-k,p]	-7.3±0.4[i-k,p]	-6.7±0.5[i-k,p]	-6.8±0.4[i-k,p]
	Phl (0.5%)[p]	-4.9±0.4[l-o]	-4.4±0.4[l-o]	-5.0±1.0[l-o]	-4.2±0.5[l-o]	-4.0±0.3[l-o]
Yellowness "b*"	Control[q]	12.3±0.9[t-w]	12.4±0.9[t-w]	15.0±0.9[t-w]	16.3±0.1[t-w]	16.4±0.3[t-w]
	AN_{100} (0.25%)[r]	15.0±0.6[t-w]	14.8±1.2[t-w]	17.6±0.7[t-w]	18.9±0.5[t-w]	18.9±0.4[t-w]
	AN_{100} (0.5%)[s]	17.8±0.7[t-w]	16.9±1.0[t-w]	19.4±1.0[t-w]	22.8±0.6[t-w]	22.8±0.6[t-w]
	AN_{80e} (0.25%)[t]	22.0±3.8[q-s,x]	22.6±3.1[q-s,x]	26.7±3.1[q-s,x]	29.9±3.2[q-s,x]	31.0±3.6[q-s,x]
	AN_{80e} (0.5%)[u]	27.3±4.2[q-s,x]	27.8±4.0[q-s,x]	23.8±3.4[q-s,x]	27.9±3.9[q-s,x]	28.3±4.4[q-s,x]
	FV_{60e} (0.25%)[v]	26.4±1.4[q-s,x]	23.8±4.0[q-s,x]	22.9±3.8[q-s,x]	17.9±2.4[q-s,x]	17.1±3.6[q-s,x]
	FV_{60e} (0.5%)[w]	30.0±3.6[q-s,x]	30.2±3.7[q-s,x]	30.3±4.1[q-s,x]	23.9±3.5[q-s,x]	23.8±3.4[q-s,x]
	Phl (0.5%)[x]	13.0±0.7[t-w]	12.8±1.4[t-w]	16.4±3.5[t-w]	13.3±0.8[t-w]	13.0±1.1[t-w]

[(a–h)] Denote a significant difference between mean values, where [a] denotes a significant difference from control and b denotes a significant difference from AN_{100} (0.25%), etc in Lightness "L*" values. [(i–p)] and [(q–x)] denote a significant difference between mean values in Greenness "-a*" values and Yellowness "b*" values, respectively. Sample abbreviations are explained in the material and methods section. Data without superscripts indicates no significant difference. One-way ANOVA followed by Tukey's post-test. n = 3 independent

3.3. Lipid oxidation in milk

Lipid oxidation during processing and storage can cause deterioration in milk quality, flavour and may also have nutritional implications. Lipid oxidation in milk can be catalysed by singlet oxygen, light or transition metals in the presence of oxygen leading to the production of a variety off-flavour breakdown products such alkanals (Fox & McSweeney, 1998). The oxidation of milk lipids is significantly reduced by the presence of naturally occurring antioxidants. Previous studies have found that iron fortification increased the rate of lipid oxidation in milk (Cook & Reusser, 1983). Seaweed extracts such as those used in the present study can contain a range of transition metals such as iron (Robledo & Pelegrin, 1997) which may increase the rate of oxidation in seaweed extract enriched milk. Lipid oxidation increased in all milk samples over the 11 day storage period (Table 3). By day 11 of storage, milk containing FV_{60e} (0.25%) and Phl (0.5%) had significantly lower ($P < 0.05$) levels of lipid oxidation compared to the other milk samples. In related studies, seaweed extracts from *Grateloupia filicina*, *Ecklonia cava* and *Fucus vesiculosus* reduced lipid oxidation in vegetable oil (Athukorala et al., 2003), fish oil (Senevirathne et al., 2006), and fish meat (Wang et al., 2010).

3.4. pH of milk

In the present study the pH of the milk samples on day 1of storage ranged from 6.52 in the control to 6.60 in the milk containing Phl (0.5%) (Table 4). By day 11 the pH of the milk had increased ranging from 6.60 in the control to 6.66 in the milk containing Phl (0.5%), however differences between samples were not significant. These results indicated that the addition of seaweed extracts to milk did not affect the pH of milk.

3.5. Microbiological analysis of milk

Pasteurisation aims to destroy the majority of vegetative pathogenic and spoilage bacteria in milk, however the presence of spore-forming bacteria such as *Bacillus* spp. and *Paenibacillus* spp. substantially limit the shelf-life of pasteurised milk (Fromm & Boor, 2006). Milk total plate counts ranged from 3.3 to 5.6 $\log_{10}$CFU over the 11 day storage period and was unaffected by the addition of seaweed extracts (Table 5). Phloroglucinol (Phl) demonstrated antimicrobial activity in milk samples. Milk was free from coliforms, yeasts and moulds on each analysis day.

The antimicrobial activity of phlorotannins (composed of phloroglucinol units) has been reported previously in a number of test systems (Eom et al., 2012) suggesting a possible food preservative role for naturally sourced, non-toxic phloroglucinol and related phlorotannins.

Table 3

Lipid oxidation in seaweed-enriched milk over 11 days at 4°C.

	Storage time at 4°C, days				
	1	4	6	8	11
Control[a]	0.014±0.02	0.009±0.01	0.010±0.02	0.019±0.03	0.025±0.01[f,h]
AN_{100} (0.25%)[b]	0.011±0.01	0.016±0.03	0.012±0.01	0.020±0.01	0.029±0.03[f,h]
AN_{100} (0.5%)[c]	0.022±0.04	0.024±0.02	0.021±0.02	0.023±0.02	0.035±0.01[f,h]
AN_{80e} (0.25%)[d]	0.012±0.03	0.013±0.05	0.016±0.03	0.010±0.01	0.023±0.04[f,h]
AN_{80e} (0.5%)[e]	0.012±0.03	0.011±0.03	0.015±0.03	0.020±0.03	0.028±0.02[f,h]
FV_{60e} (0.25%)[f]	0.022±0.04	0.016±0.02	0.015±0.03	0.021±0.01	0.018±0.03[a-e,g]
FV_{60e} (0.5%)[g]	0.018±0.01	0.017±0.01	0.022±0.03	0.026±0.03	0.028±0.03[f,h]
Phl (0.5%)[h]	0.017±0.02	0.018±0.02	0.019±0.03	0.018±0.02	0.020±0.02[a-e,g]

[(a–h)] Denote a significant difference between mean values, where a denotes a significant difference from control and b denotes a significant difference from AN_{100} (0.25%), etc. Data without superscripts indicates no significant difference. Statistical analysis was measured by one-way ANOVA followed by Tukey's post-test. n = 3 independent experiments.

Table 4
pH of seaweed-enriched milk over 11 days at 4°C.

	Storage time at 4°C, days				
	1	4	6	8	11
Control[a]	6.52±0.01	6.59±0.02	6.56±0.01	6.60±0.05	6.61±0.02
AN_{100} (0.25%)[b]	6.59±0.01	6.67±0.01	6.62±0.01	6.68±0.02	6.66±0.02
AN_{100} (0.5%)[c]	6.60±0.01	6.67±0.01	6.64±0.02	6.68±0.02	6.66±0.01
AN_{80e} (0.25%)[d]	6.57±0.01	6.65±0.01	6.61±0.01	6.64±0.02	6.61±0.01
AN_{80e} (0.5%)[e]	6.54±0.01	6.61±0.01	6.58±0.01	6.60±0.02	6.63±0.04
FV_{60e} (0.25%)[f]	6.60±0.02	6.68±0.02	6.64±0.03	6.66±0.03	6.65±0.02
FV_{60e} (0.5%)[g]	6.60±0.01	6.66±0.01	6.63±0.01	6.63±0.01	6.65±0.02
Phl (0.5%)[h]	6.60±0.01	6.68±0.00	6.64±0.01	6.66±0.01	6.66±0.01

[(a–h)] Denote a significant difference between mean values, where a denotes a significant difference from control and b denotes a significant difference from AN_{100} (0.25%), etc. Data without superscripts indicates no significant difference. Statistical analysis was measured by one-way ANOVA followed by Tukey's post-test. n = 3 independent experiments.

Table 5
Effect of seaweed extracts addition on the microbial content of milk over 11 days at 4°C.

		Storage time at 4°C, days				
		1	4	6	8	11
Control[a]	Total plate count (log_{10}CFU/ml)	3.5±0.8	3.5±0.3	4.6±0.3	4.7±1.0[h]	5.3±0.3[h]
AN_{100} (0.25%)[b]		3.5±0.5	3.4±0.5	4.8±0.5	5.0±0.3[h]	5.6±0.5[h]
AN_{100} (0.5%)[c]		3.4±0.5	3.4±0.2	4.7±0.5	4.9±0.5[h]	5.4±0.3[h]
AN_{80e} (0.25%)[d]		3.4±0.8	3.0±1.0	4.5±0.3	4.6±0.5[h]	5.3±0.5[h]
AN_{80e} (0.5%)[e]		3.3±1.0	3.4±0.3	4.6±0.3	4.4±0.3[h]	5.2±1.0[h]
FV_{60e} (0.25%)[f]		3.3±0.3	3.1±0.8	4.6±0.5	4.6±0.8[h]	5.3±1.0[h]
FV_{60e} (0.5%)[g]		3.4±0.5	3.4±0.3	4.4±1.0	4.9±0.8[h]	5.5±0.8[h]
Phl (0.5%)[h]		2.7±0.8	2.5±1.0	4.0±0.3	3.4±0.8[a-g]	3.2±0.5[a-g]
Control	Total coliforms (log_{10}CFU/ml)	nd	nd	nd	nd	nd
AN_{100} (0.25%)		nd	nd	nd	nd	nd
AN_{100} (0.5%)		nd	nd	nd	nd	nd
AN_{80e} (0.25%)		nd	nd	nd	nd	nd
AN_{80e} (0.5%)		nd	nd	nd	nd	nd
FV_{60e} (0.25%)		nd	nd	nd	nd	nd
FV_{60e} (0.5%)		nd	nd	nd	nd	nd
Phl (0.5%)		nd	nd	nd	nd	nd
Control	Yeasts and moulds (log_{10}CFU/ml)	nd	nd	nd	nd	nd
AN_{100} (0.25%)		nd	nd	nd	nd	nd
AN_{100} (0.5%)		nd	nd	nd	nd	nd
AN_{80e} (0.25%)		nd	nd	nd	nd	nd
AN_{80e} (0.5%)		nd	nd	nd	nd	nd
FV_{60e} (0.25%)		nd	nd	nd	nd	nd
FV_{60e} (0.5%)		nd	nd	nd	nd	nd
Phl (0.5%)		nd	nd	nd	nd	nd

[(a–h)] Denote a significant difference between mean values, where a denotes a significant difference from control and b denotes a significant difference from AN_{100} (0.25%), etc. Data without superscripts indicates no significant difference. Statistical analysis was measured by one-way ANOVA followed by Tukey's post-test. n = 3 independent experiments.

3.6. Sensory evaluation of milk

Sensory evaluation and instrumental data are presented in the APLSR plot in Figure 1 with the corresponding ANOVA values in Table 6. As a general rule positive and negative correlations indicate like and dislike of selected attributes, respectively.

The control milk ($P < 0.001$) and milk containing AN_{100} (0.25%) ($P < 0.01$) were positively correlated with the colour descriptor. Milk containing FV_{60e} (0.5%) was significantly negatively correlated to colour possibly due to high instrumental "b*" yellowness values (Table 2).

Milk containing AN_{80e} (0.5%) ($P < 0.05$) and FV_{60e} (0.25%) ($P < 0.05$) was significantly positively correlated with the fishy taste sensory descriptor. A similar finding was reported by Chee et al. (2005) in algal-oil enriched strawberry yogurt. The addition of seaweed extracts had no significant effect on the odour of milk. Milk flavour was essentially unaffected by the addition of seaweed extracts however milk containing AN_{80e} (0.5%) ($P < 0.05$) was negatively correlated to flavour and positively correlated ($P < 0.05$) to the off-flavour sensory descriptor. Milk containing FV_{60e} (0.5%) ($P < 0.05$) was positively correlated to the texture descriptor.

The control ($P < 0.05$) and the milk containing AN_{100} (0.5%) ($P < 0.05$) were positively correlated to overall acceptability. Milk samples containing AN_{80e} (0.5%) and FV_{60e} (0.25%) were negatively correlated to overall acceptability indicating reduced acceptability by sensory panelists (Table 6, Figure 1). Since similar negative correlations were observed for these milk samples with respect to the fishy taste descriptor, fishy taste was deemed an important factor governing the overall acceptability of seaweed extract enriched milk.

The sensory properties of milk as a function of time were also examined (Table 6 and Figure 1). Colour flavour, and overall acceptability were positively correlated to day 1 and 4 and negatively correlated to day 8 and 11. These results indicated that sensory attributes of milk decreased after day 8 of storage.

Negative sensory associations (fishy taste and off-flavour) with some of the seaweed extract enriched milk samples examined in the present study could potentially be addressed with the use of food flavourings or by micro-encapsulating the extracts in order to mask undesirable flavours associated with seaweed extracts (Petrotos et al., 2012).

Table 6
P-values of regression coefficients as derived by jack-knife uncertainty testing.

	Milk							Storage time at 4°C, days			
	Control	AN_{100} (0.25%)	AN_{100} (0.5%)	AN_{80e} (0.25%)	AN_{80e} (0.5%)	FV_{60e} (0.25%)	FV_{60e} (0.5%)	1	4	8	11
Sensory Attribute											
Colour	0.001***	0.01**	0.47ns	-0.086ns	-0.70ns	-0.99ns	-0.001***	0.001***	0.001***	-0.01**	-0.001***
Fishy taste	-0.063ns	-0.34ns	-0.05*	0.32ns	0.05*	0.05*	0.80ns	-0.001***	-0.16ns	0.001***	0.45ns
Odour	-0.93ns	-0.34ns	-0.60ns	0.56ns	0.94ns	0.81ns	0.16ns	-0.001***	-0.01**	0.01**	0.001***
Flavour	0.091ns	0.97ns	0.12ns	-0.41ns	-0.05*	-0.11ns	-0.58ns	0.001***	0.01**	-0.001***	-0.01**
Off-flavour	-0.46ns	-0.90ns	-0.05*	0.26ns	0.05*	0.09ns	0.93ns	-0.001***	-0.05*	0.001***	0.065ns
Texture	-0.21ns	-0.45ns	-0.34ns	0.92ns	0.36ns	0.77ns	0.05*	-0.01**	-0.45ns	0.86ns	0.01**
Overall acceptability	0.05*	0.85ns	0.05*	-0.20ns	-0.05*	-0.05*	-0.31ns	0.001***	0.01**	-0.001***	-0.001***
Instrumental and chemical analysis											
TBARS	-0.001***	-0.05*	-0.001***	0.001***	0.19ns	0.01**	0.001***	-0.001***	-0.001***	0.001***	0.001***
pH	-0.001***	-0.001***	-0.001***	0.001***	0.001***	0.001***	0.01**	-0.001***	-0.001***	0.001***	0.001***
"L*" values	0.001***	0.001***	0.001***	-0.001***	-0.001***	-0.30ns	-0.001***	0.21ns	0.001***	-0.001***	-0.75ns
"a*" values	0.001***	0.001***	0.001***	-0.001***	-0.001***	-0.05*	-0.001***	0.01**	0.05*	-0.84ns	-0.97ns
"b*" values	-0.001***	-0.001***	-0.001***	0.001***	0.001***	0.01**	0.001***	-0.86ns	-0.001***	0.01**	0.14ns
DPPH scavenging activity	-0.001***	-0.001***	-0.001***	0.001***	0.001***	0.001***	0.001***	-0.33ns	-0.21ns	0.01**	0.001***

Significance of regression coefficients; ns, not significant, *95% significance, $P < 0.05$, **99% significance, $P < 0.01$, ***99.9% significance, $P < 0.001$. n = 3 independent experiments.

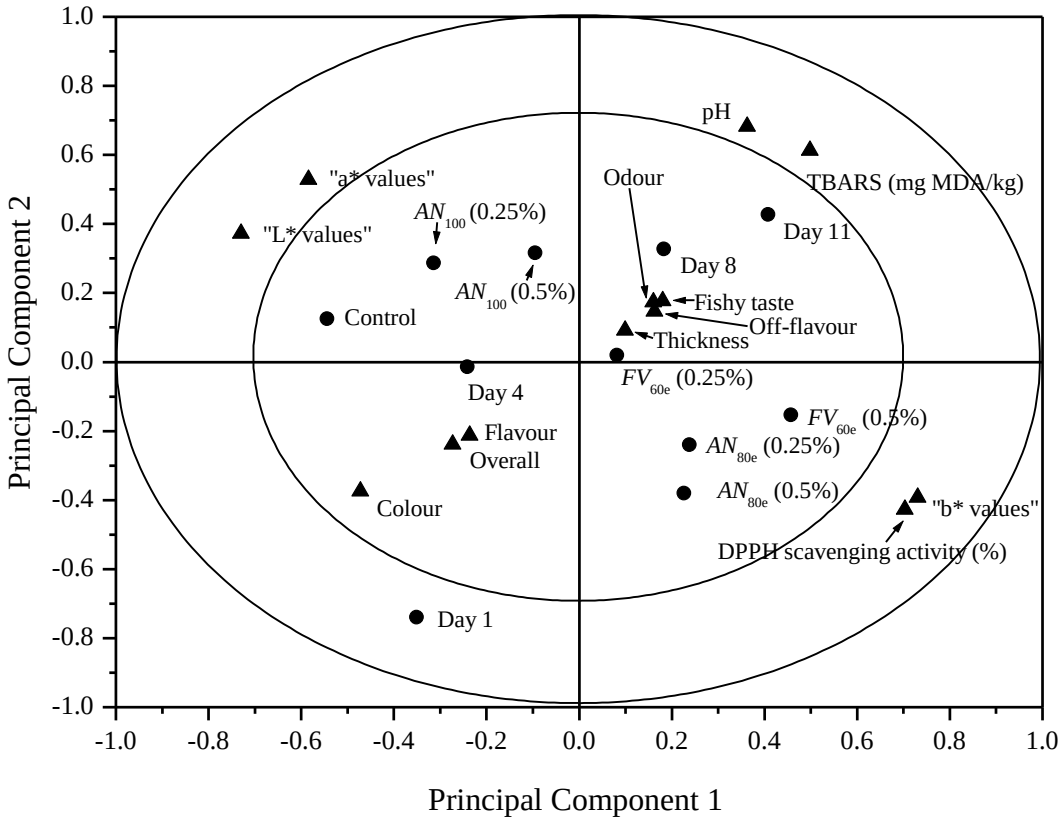


Figure 1. APLSR for the various seaweed-enriched milk samples. ▲=Milk sample, ●=sensory descriptor, chemical and instrumental variables. The concentric circles represent 100% and 50% explained variance, respectively.

3.7. Stability of seaweed extracts in milk

Previous studies have found that antioxidant activity of antioxidant enriched dairy products decreased over of time (Ilic & Ashoor, 1988; Najgebauer-Lejko et al., 2011). The DPPH radical scavenging activity of all milk samples was stable over the 11 day storage period (Figure 2). All seaweed-enriched milk samples had significantly ($P < 0.05$) higher DPPH radical scavenging activity than the non enriched milk control. The DPPH radical scavenging activities of all enriched milk samples was unaffected by seaweed extract and type or concentration. These findings suggested that the seaweed extracts may be used as stable antioxidant ingredients in milk and other dairy products.

3.8. *In-vitro* antioxidant activity of milk and milk digestates

The activity of many antioxidant compounds such as polyphenols may be altered following *in-vitro* digestion due to pH change and enzymatic hydrolysis (Stalmach et al., 2012). Therefore the antioxidant activity of seaweed enriched milk samples was assessed before and after an *in-vitro* digestion procedure by determining the DPPH radical scavenging activity and the ferrous iron chelating activity. The potential antioxidant activity of the milk samples were further investigated by assessing their protective against H_2O_2-induced DNA damage in Caco-2 cells. The milk supplemented with Phl (0.5%) had the highest DPPH activity which did not significantly ($P < 0.05$) change after digestion (Figure 3). The DPPH radical scavenging activity of the milk containing AN_{80e} (0.5%) significantly decreased after digestion. The scavenging activity of all the other milk samples was similar pre and post-digestion.

The FICA activity of the undigested milk samples ranged from 22.5% in the control milk to 58.5% in the Phl (0.5%) milk sample. After digestion, the FICA of all samples significantly increased ranging from 77.5% in the control and sample containing FV_{60e} to 92.5% in the milk containing Phl (0.5%) (Figure 4) and this suggests that digestion enhances the antioxidant potential of samples and therefore potentially increases their potential health benefits. Similar increases in FICA were observed in yogurts digestates (Chapter 6) and was attributed to the presence of milk peptides and iron-chelating components such as polyphenols from seaweed extracts released during the *in-vitro* digestion procedure (Hurrell et al., 2006).

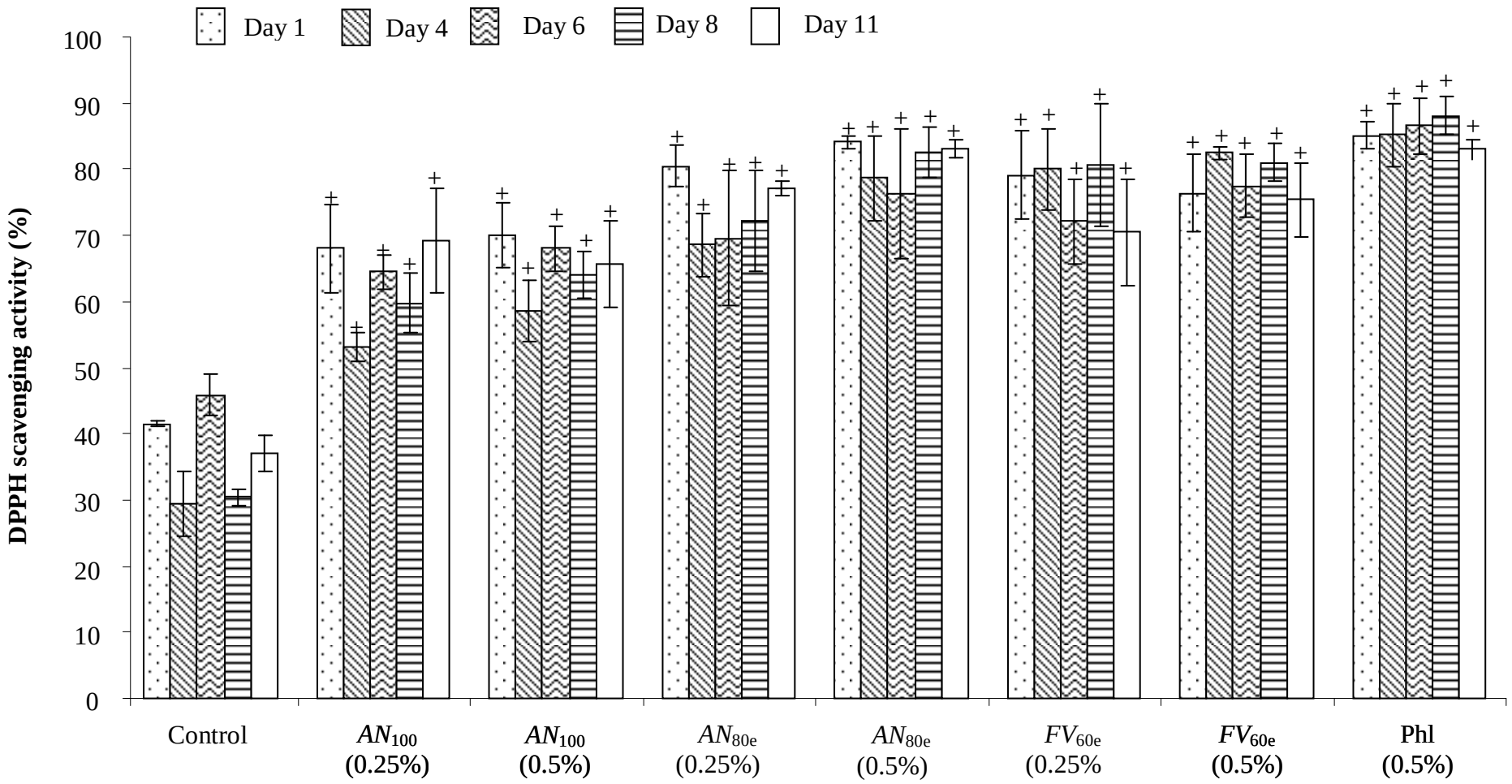


Figure 2. The DPPH-scavenging activity % (stability) of milk samples over 11 days storage at 4°C. Data are the means of three independent experiments, with standard errors represented by vertical bars. $^{+}$ denotes significantly higher ($P < 0.05$) DPPH radical scavenging activity, on each measurement day, between seaweed extract enriched milk samples and the corresponding controls.

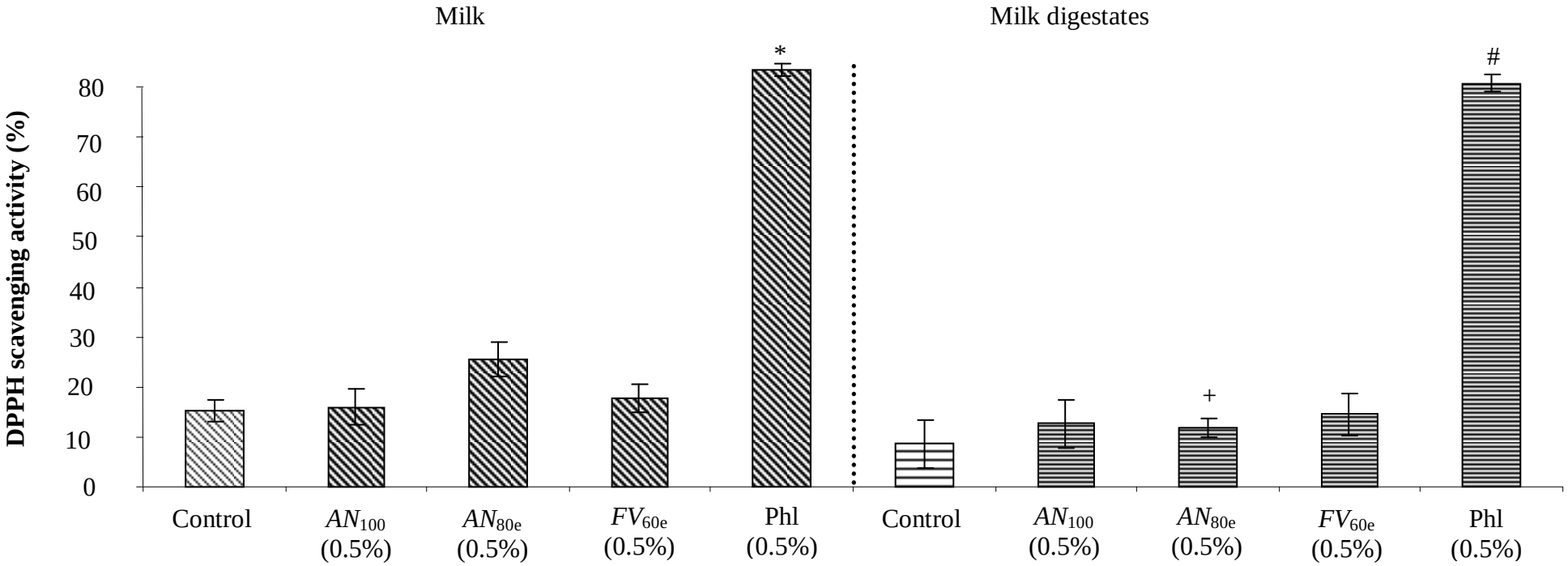


Figure 3. The DPPH-scavenging activity of milk samples before and after an *in-vitro* digestion procedure. Data are the means of four independent experiments, with standard errors represented by vertical bars and determined using repeated measures ANOVA followed by Tukey's post-test. * and # denotes significantly higher DPPH scavenging activity values between milk samples and milk digestates, respectively. + denotes significant different DPPH scavenging activity values between different a milk sample and its corresponding digestate.

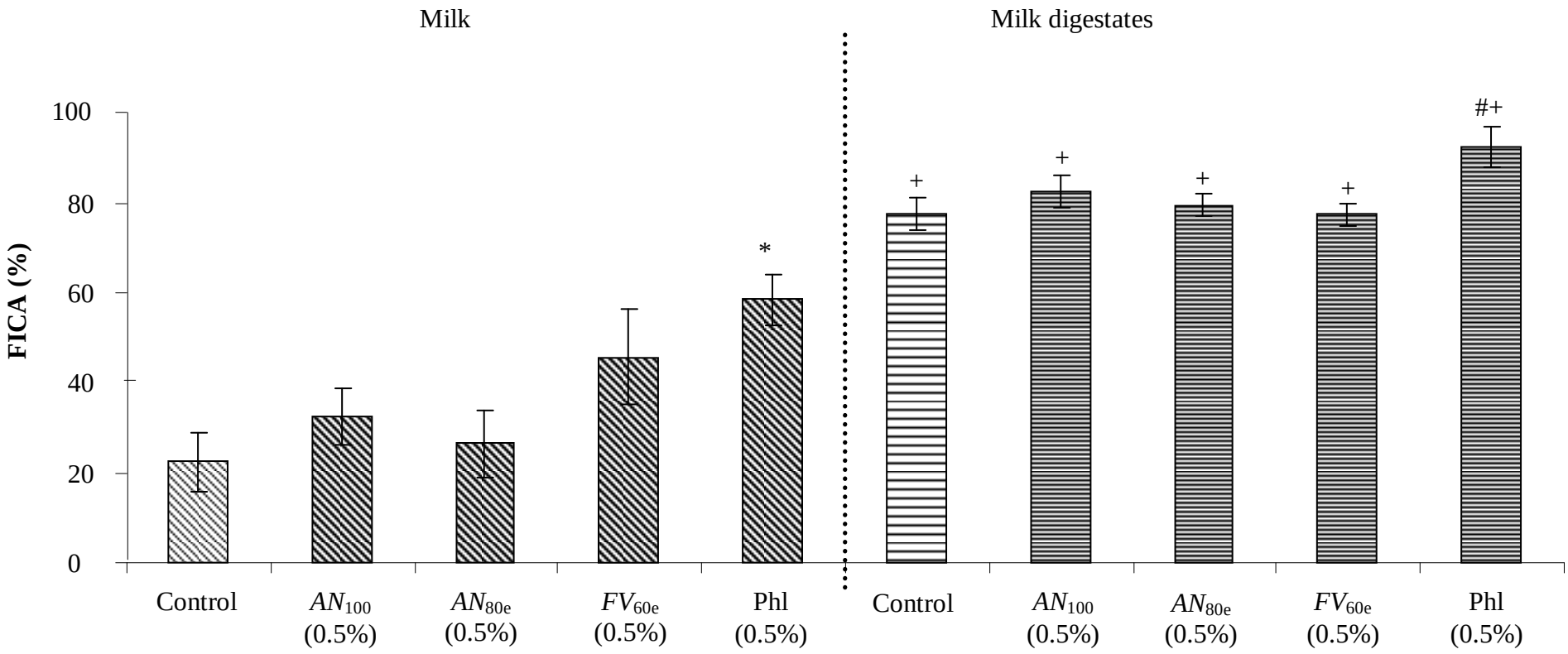


Figure 4. The ferrous-ion chelating activity (FICA) (%) of milk samples before and after an *in-vitro* digestion procedure. Data are the means of four independent experiments, with standard errors represented by vertical bars. * and # denotes significantly higher FICA between milk samples and milk digestates, respectively. + denotes significantly different FICA between different a milk sample and its corresponding digestate.

3.9. Cellular antioxidant activity of milk and milk digestates

The potential cytotoxicity of each of the milk extracts at concentrations ranging from 0 to 5 mg/ml was assessed in Caco-2 cells using the neutral red assay. Non toxic concentrations of the digested and undigested milk samples were determined using the neutral red assay and milk were added to Caco-2 cells at 5 mg/ml media (0.025 mg seaweed extract) and digestates at 0.33 mg/ml media (0.0016 mg seaweed extract) (Table 7). Cellular antioxidant effects were examined by measuring the SOD and CAT activities and the GSH content in Caco-2 cells exposed to seaweed extract enriched milk and milk digestates for 24 hrs. It was found that the addition of seaweed extract enriched milk or digestates did not significantly alter the antioxidant status in Caco-2 cells (Table 8). The DNA protective effects of the seaweed supplemented milk and digestates against H_2O_2 induced DNA damage in Caco-2 cells was determined using the alkaline single cell gel electrophoresis assay (comet assay). Caco-2 cells were pre-incubated with milk and milk digestates for 24 hrs and DNA damage was induced by the addition of H_2O_2 (50 µM) which increased DNA damage from a control level of 10% to 60% (Table 9). None of the milk or digestate samples protected against DNA damage in Caco-2 cells under the conditions employed in the present study.

Previous studies have found that crude extracts and purified compounds from seaweed protected against H_2O_2-induced DNA damage in cells (Kang et al., 2011; O'Sullivan et al., 2011) however, at similar seaweed concentrations, the seaweed supplemented milk samples did not demonstrate antioxidant effects in Caco-2 cells. Previous studies have reported that, in the presence of milk proteins, the DPPH radical scavenging and antioxidant activity of a number of polyphenol compounds was reduced due to the formation of polyphenol-milk protein complexes (Xiao et al., 2011; Lorenz et al., 2007; Ryan & Petit, 2010). The lack of cellular antioxidant activity in digested milk samples may be due to pH-mediated modification of compounds present in the seaweed extracts during digestion.

Conclusion

The addition of seaweed extracts to milk influenced certain milk quality and shelf-life attributes. Ethanolic extracts from *Ascophyllum nodosum* and *Fucus vesiculosus* increased the greenness and yellowness of fortified milk samples. Seaweed extracts may contain pro-oxidant components (eg iron) in addition to polyphenolic

antioxidants. Ethanolic extracts from *Fucus vesiculosus* (FV_{60e}) displayed antioxidant functionality in milk similar to the phloroglucinol standard. Quality parameters such as pH and microbiology was unaffected by seaweed extract type or concentration. Sensory analysis indicated that water prepared extracts were more acceptable than ethanolic extracts as functional ingredients in milk. Seaweed extracts were stable in milk and displayed varying degrees of antioxidant activity (DPPH and FICA) pre and post *in-vitro* digestion. Seaweed enriched milk did not exhibit cellular antioxidant activity indicating reduced biological activity of the extracts in milk.

Further research is necessary to examine the potential of seaweed extracts in typical functional fermented dairy products such as yogurt.

Table 7

Viability of Caco-2 cells following addition of increasing levels (ml per ml media) of seaweed extract-enriched milk.

	Weight of milk added, mg			
	0	0.5	2.5	5.0
Undigested samples		% viability		
Control	100	100	100	100
AN_{100} (0.5%)	100	100	100	95.5
AN_{80e} (0.5%)	100	100	100	100
FV_{60e} (0.5%)	100	100	100	85.0
Phl (0.5%)	100	100	100	92.5
	Weight of milk added, mg			
	0	0.3	0.67	1.0
Digested samples		% viability		
Control	100	100	100	100
AN_{100} (0.5%)	100	91.5	100	100
AN_{80e} (0.5%)	100	89.5	100	100
FV_{60e} (0.5%)	100	95	100	100
Phl (0.5%)	100	92.5	100	100

Mean values ± SEM. n = 3 independent experiments.

Table 8

The effect of seaweed-enriched milk and milk digestates on the antioxidant status of Caco-2 cells.

	SOD activity (% Control)	CAT activity (% Control)	GSH content (% Control)
Undigested samples			
AN_{100} (0.5%)	100.1 ± 7.5	95.5 ± 1.5	100.9 ± 5.5
AN_{80e} (0.5%)	97.0 ± 6.5	95.5 ± 2.5	104.5 ± 10.5
FV_{60e} (0.5%)	91.5 ± 8.0	93.5 ± 7.0	110.6 ± 5.5
Phl (0.5%)	108.0 ± 8.5	86.5 ± 3.5	108.0 ± 9.5
Digested samples			
AN_{100} (0.5%)	100.5 ± 7.0	95.5 ± 15	106.6 ± 11.5
AN_{80e} (0.5%)	97.0 ± 6.5	95.5 ± 5.5	110.5 ± 5.0
FV_{60e} (0.5%)	93.5 ± 8.0	93.5 ± 7.0	105.5 ± 10.5
Phl (0.5%)	108.0 ± 8.5	96.5 ± 3.5	108.0 ± 9.5

Mean values ± SEM. n = 3 independent experiments

Table 9

The potential protective activity of seaweed-enriched milk and milk digestates against H_2O_2-induced DNA damage in Caco-2 cells.

	Tail DNA (%)
Undigested samples	
Control	10
H_2O_2 control	60
AN_{100} (0.5%)	55.5 ± 7.5
AN_{80e} (0.5%)	57.0 ± 6.5
FV_{60e} (0.5%)	59.5 ± 8.5
Phl (0.5%)	58.0 ± 8.0
Digested samples	
AN_{100} (0.5%)	59.5 ± 7.5
AN_{80e} (0.5%)	55.0 ± 5.5
FV_{60e} (0.5%)	63.5 ± 8.5
Phl (0.5%)	66.0 ± 4.0

Mean values ± SEM. n = 3 independent experiments

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CHAPTER 6

An investigation of the performance of seaweed extracts as potential functional ingredients in yogurt.

Abstract

Extracts were manufactured from *Ascophyllum nodosum* with 100% water (AN_{100}) and 80% ethanol (AN_{80e}) and *Fucus vesiculosus* with 60% ethanol (FV_{60e}) using solid-liquid extraction techniques. Yogurts containing seaweed extracts (AN_{100}, AN_{80e} and FV_{60e}) at 0.25 and 0.5% (w/w) were manufactured and stored for up to 28 days at 4°C. Yogurt composition, quality and shelf-life parameters, stability and bioactivity of seaweed extracts in yogurt was examined over the 28 day storage period. Yellowness 'b*' values were significantly ($P < 0.05$) higher in yogurts containing FV_{60e} (0.25%), FV_{60e} (0.5%) and AN_{80e} (0.5%). Yogurts containing AN_{80e} (0.5%) and FV_{60e} (0.5%) had lower levels of lipid oxidation compared to the other yogurt samples. The pH, microbiology and whey separation in yoghurt were unaffected by seaweed extract addition. The modulus of yogurt was higher in the control compared to seaweed extract enriched yogurts. Sensory analysis indicated that the control yogurt and yogurts containing AN_{100} (0.25%) and AN_{100} (0.5%) were the most acceptable by panellists. The antioxidant activity (DPPH radical scavenging activity) of seaweed extracts in yogurt was stable as a function of storage time. Yogurt (undigested) or yogurt digestates did not affect the antioxidant status (CAT, SOD and GSH activity) or protect against H_2O_2-induced DNA damage in Caco-2 cells. Results indicate that certain seaweed extracts may be incorporated into yogurt without negatively affecting yogurt quality and shelf-life. Seaweed extracts did not display bioactivity when incorporated into yogurt as functional ingredients.

1. Introduction

Yogurt and related products are some of the most commonly manufactured food products worldwide with expected production rates of approximately 8,500 tonnes expected to be produced in the EU during 2013 (EU market outlook report, 2008). From a nutritional perspective, yogurt is widely perceived as a healthy food as it contains protein, riboflavin, vitamins B_6 and B_{12}, and calcium. Additionally, in recent years yogurt and other dairy products have been used as carriers for functional food ingredients, or nutraceuticals. Nutraceuticals are defined as food components which demonstrate physiological benefits or reduced risk of chronic disease beyond their basic nutritional function (Shah, 2001) while a functional food is a food that beneficially affects one or more target functions in the body beyond adequate nutritional effects in a way that is relevant to either improved state of health and well- being and/or reduction in disease risk (Roberfroid, 2000). The main difference between nutraceuticals and functional foods is that nutraceuticals are sold in dosage form.

Bifidobacterium and *Lactobacillus*-enriched probiotic yogurt is among the most common type of functional food products marketed heavily worldwide. Yogurt is also used commercially as a carrier for gut-friendly prebiotics (Thomas & Greer, 2010) and cholesterol reducing phytosterols (Moreau, 2004). Laboratory scale studies have investigated yogurt as a possible carrier vehicle for other functional food ingredients such as omega-3 fatty acids, vitamins and minerals (Hekmat & McMahon, 1997; Achanta et al., 2007; Let et al., 2007; Brignac & Aryana, 2012).

Antioxidants such as polyphenols and carotenoids are popular functional ingredients which exhibit a range of health benefits including anti-cancer, eye protective, heart protective and anti-diabetic properties (Pandey & Rizvi, 2009). Studies have investigated yogurt as a potential carrier vehicle for antioxidant compounds with the aim of increasing the health benefits and quality attributes of the resulting product. Cossu et al. (2009) produced yogurts enriched with crude extracts from artichoke (*Cynara scolymus* L.), strawberry-tree fruit (*Arbutus unedo* L.) and cherry (*Prunus avium* L.) and found that the fortified yogurts exhibited significantly higher antioxidant activity (total phenol content (TPC), FRAP and DPPH scavenging activities) compared to the non-enriched yogurt. Similarly green and Pu-erh tea infusions (5-15%) increased the antioxidant capacity (FRAP and DPPH scavenging

activities) of enriched yogurt (Najgebauer-Lejko et al., 2011). Other studies have produced yogurt containing polyphenols from apple (Sun-Waterhouse et al., 2011) and pycnogenol from Maritime Pine (Ruggeri et al., 2008) and reported increased TPC in yogurt. In additional studies, yogurts were manufactured containing antioxidant rich extracts from carrot juice (Salwa et al., 2004), pomegranate (Kale et al., 2007) and olive fruit (Petrotos et al., 2012), however investigations were limited to physicochemical and sensory attributes of yogurts where antioxidant activity was not determined.

A number of seaweed and seaweed-derived extracts have demonstrated superior antioxidant activity compared to terrestrial plants (Budhiyanti et al., 2011). The antioxidant activity of both crude and purified seaweed extracts has been reported in *in-vitro, in-vivo*, and in food models. O'Sullivan et al. (2011) demonstrated that crude extracts from a range of brown seaweeds exhibited *in-vitro* antioxidant activity and DNA protective effects against H_2O_2-induced DNA damage in Caco-2 cells. Kang et al. (2011) observed significantly potent DNA protective activity by a purified compound (pyrogallol-phloroglucinol-6,6'-bieckol) from *Ecklonia cava* against H_2O_2-induced damage in a kidney fibroblast cell line (Vero cell). Kim et al. (2008) reported enhanced cellular catalase (CAT) and glutathione peroxidise (GPx) activity and reduced lipid oxidation in the erythrocytes of diabetic patients following the consumption of a combination of crude extracts from *Laminaria japonica* and *Undaria pinnatifida*. Wang et al. (2010) found that the addition of crude and chromatographically purified extracts from *Fucus vesiculosus* significantly reduced the rate of lipid oxidation in cod fish muscle.

Additional marine-derived ingredients used to improve yogurt quality include algal oil which resulted in an omega-3 fatty acid enriched yogurt product (Chee et al., 2009). To date, no studies investigating the addition of seaweed extracts to dairy products as a means of enhancing yogurt antioxidant activity have been reported in the scientific literature. The antioxidant activity of a foodstuff may be altered during the digestive process as certain antioxidant compounds can be degraded by pH changes and digestive enzymes while others may be released from the food matrix (Nagah & Seal, 2005).

In previous studies carried out in our research group extracts from *Ascophyllum nodosum* (100% H_2O (AN_{100}) and 80% ethanol (AN_{80e}) extracts) and *Fucus vesiculosus* (60% ethanol extract (FV_{60e}) exhibited a range of *in-vitro* and cellular

antioxidant activities (Chapter 4). In chapter 5 milk was enriched with AN_{100}, AN_{80e} and FV_{60e} at concentrations of 0.25 and 0.5% (w/w). The objective of this study was to manufacture yogurt containing AN_{100}, AN_{80e} and FV_{60e}. The effect of seaweed extracts (0.25 and 0.5%) (w/w) on the quality and shelf-life (composition, colour, lipid oxidation, pH, microbiology, whey separation and rheology) and sensory characteristics of yogurts was investigated over a 28 day storage period. The stability of seaweed extracts in yogurt was assessed using the DPPH radical scavenging activity assay. Seaweed extract enriched yogurts were subjected to an *in-vitro* digestion procedure to assess the antioxidant capacity of the yogurts before and after digestion. Antioxidant activity of the yogurt and yogurt digestates was determined using *in-vitro* antioxidant (DPPH radical scavenging activity and ferrous-chelating activity (FICA)) and cellular antioxidant (Catalase (CAT), superoxide dismutases (SOD) and glutathione (GSH)) assays. The ability to protect against oxidant-induced DNA damage in human adenocarcinoma Caco-2 cells was also investigated.

2. Materials and Methods

2.1. Materials

Fresh whole milk was obtained from a local retail outlet. Yogurt culture (Yo-flex) was obtained from CHR Hansen (Cork). Human colon adenocarcinoma Caco-2 cells were purchased from the European Collection of Animal Cell Cultures (Salisbury, UK). Foetal bovine serum (FBS) was purchased from Invitrogen (Paisley, Scotland). Cell culture plastics were purchased from Cruinn Diagnostics (Greiner Bio-One, Frickenhausen, Germany). Calbiochem superoxide dismutases (SOD) Assay Kit II and Catalase (CAT) kit was purchased from Merck Chemicals Ltd. (Nottingham, UK). Agar was purchased from Oxoid Ltd., Basingstoke, Hampshire, England. All other cell culture chemicals and reagents were purchased from Sigma-Aldrich Ireland Ltd., Arklow, Co. Wicklow, Ireland.

2.2. Preparation and characterisation of seaweed extracts

Ascophyllum nodosum was collected from Finnavara, Co. Clare, Ireland. *Fucus vesiculosus* was harvested from Spiddal, Co. Galway, Ireland. Seaweeds were freeze-dried at -20˚C for 72 hrs, vacuum-packed and stored at -80ºC prior to extraction. Seaweed extracts were manufactured from freeze-dried seaweed using solid-liquid extraction and one of three solvent systems at room temperature: water (100%) and ethanol/water (80 : 20) for *Ascophyllum nodosum* (AN_{100} and AN_{80e}) and ethanol/water (60 : 40) for *Fucus vesiculosus* (FV_{60e}).

Dried seaweed (250 g) was ground to a fine powder using a blender (Waring Laboratory Science, USA). In water (100%) and ethanol-based extractions (80 : 20 or 60 : 40 ethanol/water) ground seaweed was suspended in the extracting solvent at ratios of 1 : 20 and 1 : 10 (w/v), respectively. Water and ethanol extractions were carried out by incubation in an orbital shaker (MaxQ 6000 Shaker, Thermo Fisher Scientific, Ireland) at 175 rpm. Water extracts were filtered after 6 hrs incubation, the seaweed material was re-suspended in water (1 : 20 (w/v)) and filtered after 24 hrs through glass wool filters due to the viscosity of the resulting extract. The water extracts were combined and concentrated by rotary evaporation (Buchi Rotavapor R-220, Mason Technologies, Ireland) at 50ºC.

Ethanol extracts were filtered through a Buchner funnel after 3 hrs incubation and the seaweed material was re-suspended in ethanol/water (1 : 10 (w/v)) for an additional 3 hrs. This process was repeated with a final overnight extraction to

ensure exhaustive extraction occurred. The solvent extracts were combined and ethanol was removed by rotary evaporation at 50°C. All seaweed extracts (AN_{100}, AN_{80e} and FV_{60e}) were subsequently freeze-dried, ground and stored at -80ºC prior to analysis.

The antioxidant activity of AN_{100}, AN_{80e} and FV_{60e} was determined using *in-vitro* antioxidant assays (total phenol content (TPC), DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate) radical scavenging activity and ferrous-ion chelating activity (FICA)) assays.

2.3. *In-vitro* antioxidant assays

2.3.1. Total phenol content (TPC)

The TPC of the seaweed extracts was measured according to the method of Singleton & Rossi (1965). Gallic acid was used as a standard and TPC was expressed in terms of mg gallic acid equivalents/g dry weight (GAE/g).

2.3.2. DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate) radical scavenging activity

Seaweed extracts were dissolved in methanol at a concentration of 1 mg/ml. Seaweed extracts (500 µl) were added to 3.5 ml DPPH solution (in methanol) (100 µM) and mixed vigorously using a vortex mixer. Samples were stored in the dark for 60 min and subsequently centrifuged (Sigma 4K15, Sigma Centrifuge GmbH, Osterode am Harz, Germany) at 3000g for 10 min at room temperature. The absorbance of the supernatant was measured at 515 nm using a spectrophotometer (WPA lightwave, Biochrom, Cambridge, UK). Trolox (0.04 µM) was used as a standard for comparative purposes. A control was prepared containing DPPH and methanol only. Data was expressed as % DPPH radical scavenging activity and calculated as follows:

$$\text{DPPH activity, \%} = (1 - (\text{Abs}_{515nm}\ \text{Seaweed extract} / \text{Abs}_{515nm}\ \text{Control})) \times 100$$

2.3.3. Ferrous-ion chelating activity

Ferrous-ion chelating activity **(**FICA) was carried out as described by Wang et al. (2009). In a 96-well plate 100 µl of each seaweed extract (1 mg/ml) were mixed with 150 µl distilled water and 5 µl $FeCl_2$ (2 mM). The reaction was initiated by the addition of 5 µl ferrozine (5 mM). Following 10 mins incubation at room temperature the absorbance was measured at 562 nm using a plate reader (Thermo

Scientific Varioskan Plate Reader, Fisher Scientific UK Ltd). The assay positive control contained distilled water and all assay reagents. The colour blank (containing seaweed extracts and all reagents apart from ferrozine) was used to correct the colour of the seaweed extracts. The % ferrous ion-chelating activity (FICA) was calculated as follows:

FICA, % = (1 - ((Abs_{562nm} Seaweed extract - Abs_{562nm} Colour blank) /Abs_{562nm} Control)) × 100

2.4. Addition of seaweed extracts to milk and yogurt manufacture

Prior to yogurt manufacture, seaweed extracts (AN_{100}, AN_{80e} and FV_{60e}) were added to milk (1000 ml) at concentrations of 0.25% and 0.5% (w/w) and mixed for 5 hrs at 4°C with a magnetic stirrer to aid dissolution. Seaweed extract-containing milk was heated in a waterbath until 93°C was reached and then held at this temperature for 15 minutes to pasteurise the milk and partially denature the whey proteins present, and subsequently cooled to 43°C. Yogurt culture (Yo-flex, CHR Hansen) was added at a concentration of 0.1% (v/v) and milk samples were further incubated at 43°C until a pH of 4.5 was reached. Yogurt samples were subsequently stirred to break the gel network and ~ 95g portions were packaged aseptically in 100 ml sterile containers (Sarstedt Ltd., Co. Wexford, Ireland) and stored for 28 days at 4°C.

2.5. Compositional analysis

The moisture and fat content of yoghurt were measured using the SMART Trac rapid moisture/fat analyser (CEM Corporation, NC, USA). Protein nitrogen was determined by the Kjeldahl method of the Association of Official Analytical Chemists (AOAC, 1995). Total protein was calculated by multiplying the protein nitrogen by a factor of 6.38. The ash content was determined using a muffle furnace (AOAC, 1995). The carbohydrate content was calculated by difference. The composition of commercially available natural yoghurt was also analysed for comparative purposes. Compositional analysis results were expressed as percentage values, %.

2.6. Surface colour measurement of yogurt

The surface colour of yogurt samples was measured using a Minolta CR-400 colorimeter (Minolta Camera, Co. Ltd., Japan). The chroma meter consisted of a

measuring head (CR-400), with an 8 mm diameter measuring area, a 2° standard observer, and a data processor (DP-400). The chroma meter was calibrated using the CIE Lab colour space system using a white tile (C: Y = 93.6, x = 0.3130, y = 0.3193) and Yxy values were converted to L*a*b*. The 'L*' value represents lightness and '-a*' and 'b*' values represent greenness and yellowness, respectively. Six measurements were recorded and an average value was reported per yogurt sample. Colour measurements were recorded at 7 day intervals up to 28 days of storage.

2.7. Measurement of lipid oxidation in yogurt

Lipid oxidation in yoghurt was measured following the procedure of Fenaille et al. (2001) with modifications. Yoghurt (5 g), 10% trichloroacetic acid (TCA) (5 ml) and distilled water (5 ml) were added to a 50 ml screw capped plastic tube (Sarstedt Ltd., Wexford, Ireland) and the contents were shaken vigorously. The tubes were centrifuged at 2700g for 5 min at room temperature (Sigma 4K15). The supernatant was subsequently filtered through Whatman No.1 filter paper. In a screw cap test tube, 4 ml of clear filtrate was added to 1 ml of 0.06M TBA reagent (dissolved in water). The tubes were placed in a water bath (Model B21, Fisher Scientific Ltd., Dublin, Ireland) and held at 70°C for 1 hr. Following cooling, the absorbance of the TBA-malondialdehyde complex was measured on a spectrophotometer (Cary 300 Bio, UV-Vis spectrophotometer, Varian Instruments, CA, USA) at 532 nm against a blank containing all reagents (2 ml distilled water, 2 ml 10% TCA and 1 ml 0.06 M TBA reagent). The malondialdehyde content of yoghurt was calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. Results were expressed as 2-thiobarbituric acid-reactive substances (TBARS) in mg malondialdehyde (MDA)/kg yoghurt. Lipid oxidation in yogurt samples was measured at 7 day intervals up to 28 days of storage.

The percentage recovery of a malondialdehyde standard (TEP, 1,1,3,3-tetraethoxypropane) was determined in the presence of yoghurt. TEP (1 ml) at two concentrations, 20 and 40 nmoles/ml, was added to yoghurt (5 g) in addition to 10% TCA (5 ml) and distilled water (4 ml) as described above. Following centrifugation and filtration, the filtrate (4 ml) was incubated with TBA reagent (0.06 M) and the absorbance at 532 nm was compared against the absorbance of TEP (1 ml) (20 and 40 nmoles/ml) diluted to 15 ml with distilled water and incubated with TBA. The absorbance of the yoghurt sample filtrate reacted with TBA reagent was subtracted

from the absorbance of the yoghurt sample filtrate containing TEP and reacted with TBA reagent. The % recovery was calculated as follows:

Recovery, % = ((Abs_{532nm} Filtrate – Abs_{532nm} Yoghurt sample) / Abs_{532nm} TEP) × 100

The % recovery of malondialdehyde in yoghurt was 95.7 ± 1.6%.

2.8. pH and microbiology of yogurt

The pH of the yogurts was measured using a pH meter (Seven Easy portable, Mettler-Toledo GmbH, Switzerland) by directly inserting the pH probe into the yogurt samples.

Yogurt samples (10 g) were transferred into stomacher bags, diluted with 90 ml of Ringer's solution and stomached for 1 min (Steward Stomacher 400 Lab Blender, London, UK) resulting in a 1/10 dilution used for analysis. Serial dilutions were prepared and colony counts were determined using the pour plate technique with MRSA (de Man, Rogosa and Sharpe agar) and M17 agar (supplemented with sterile lactose solution (10% w/v)) (Oxoid Ltd.) which assess *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* counts, respectively. The M17 plates were placed in heat-sealed bags in the presence of Anaerocult® A (Merck Millipore, Germany) to create an anaerobic environment. The MRSA plates did not require an anaerobic environment. The MRSA and M17 plates were incubated at 37°C for 4 days. Results were expressed as log_{10}CFU (colony forming units)/g yogurt. pH and microbiological analysis of yogurts was carried out at 7 day intervals up to 28 days of storage.

2.9. Whey separation

Whey separation in yogurt was assessed according to the method of Keogh & Kennedy (1998). Yogurt (15 - 20 g) was centrifuged at 3000g at 4°C for 10 min. Sample was weighed and then the clear supernatant was removed and the sample was weighed again. The % whey separation was measured as follows:

Whey separation, %, = ((Original weight of yogurt - weight of yogurt after whey removal) / original weight of yogurt) × 100

Whey separation was recorded at 7 day intervals up to 28 days of storage.

2.10. Rheology

The rheological assessment of the yogurt samples was carried out using a rheometer (Brookfield RS, Lab Unlimited, Dublin, Ireland) with a V-40/20 vane spindle attachment. Yogurt samples were removed from refrigerator immediately prior to rheology measurements. The spindle was directly inserted into each yogurt sample and a shear rate of 0.05 s^{-1} was applied to each sample for 2 min and the shear modulus (*G*) was measured. The shear modulus (*G*) of the yogurts was recorded at 7 day intervals up to 28 days of storage.

2.11. Sensory analysis

Sensory analysis of yogurt was performed using 26 naive untrained assessors (20 – 40 years age range) at 7 day intervals up to 28 days of storage. Sensory analysis descriptors were colour, texture, odour, flavour, off-flavour, thickness and overall acceptability. Assessors were asked to indicate their degree of liking on a 10 cm line scale ranging from 0 (extremely dislike) to 10 (extremely like).

Sensory analysis was undertaken in the panel booths of the university sensory laboratory which conform to the ISO (1988) international standard. Seven coded samples were given to each panellist and they were required to rinse their mouths with water before tasting each sample. The order in which the samples were presented was randomised to prevent potential flavour carryover effects (MacFie et al., 1989).

2.12. Stability and bioactivity of seaweed extract enriched yogurt

2.12.1. Stability of seaweed extracts in yogurt

The stability of seaweed extracts in yogurt samples over the storage period was determined using the DPPH radical scavenging activity assay as described in section 2.3.2. Yogurt samples were diluted in methanol (1:10) and 500 µl was added to 3.5 ml DPPH solution (100 µM) and measured as described above. The % DPPH radical scavenging activity (stability) of yogurts was measured at 7 day intervals up to 28 days of storage.

2.12.2. *In-vitro* digestion of yogurt

In-vitro digestion of yogurt samples was carried out following the method of Daly et al. (2010) with modifications. All experiemental work was carried out in UV-light

free conditions to reduce the possible photo-decomposition of the antioxidant extracts present in the yogurt. Yogurt samples (containing 0.5% AN_{100}, AN_{80e} and FV_{60e}) (1 g) were dissolved in 10 ml HBSS and shaken vigorously. Aliquots (5 ml) were transferred to amber bottles. To mimic the gastric phase of digestion, 100 µl pepsin (0.04 g/ml in 0.1 N HCl) was added to each bottle and the pH was adjusted to 2 using 1 N HCl. Oxygen was removed by blowing nitrogen over the samples. Samples were incubated at 37ºC for 1 hr in an orbital shaking (95 rpm) water bath (Grant OLS200, Keison Products, Essex, UK). Following gastric digestion, the pH was increased to 5.3 using sodium carbonate (0.9 M $NaHCO_3$) followed by the addition of 200 µl bile salts (1.2 mg/ml glycodeoxycholate, 0.8 mg/ml taurocholate and 1.2 mg/ml taurodeoxycholate) and 100 µl pancreatin (0.08 g/ml HBSS). The pH was subsequently adjusted to 7.4 using NaOH, oxygen was displaced by nitrogen and samples were incubated at 37°C in the orbital shaking water bath for a further 2 hrs. Following the simulated intestinal digestion, the digested milk samples were ultracentrifuged (Beckman L7–65 ultracentrifuge; Palo Alto, CA, USA) at 223,487 g for 95 min to isolate the micelle (aqueous) fraction. Digestion samples were filter sterilised using 0.22 µm syringe-driven filters (Merck-Millipore, Cork).

2.12.3. Effect of digestion on the antioxidant activity of seaweed enriched yogurts

Yogurt and subsequent digestions were diluted in water or methanol (1:10) for FICA and DPPH radical scavenging activity, respectively (section 2.3).

2.12.4. Cellular antioxidant activity of yogurt and yogurt digestates

Human colon adenocarcinoma Caco-2 cells were maintained as described by O'Sullivan et al. (2011). Caco-2 cells were supplemented with increasing concentrations (0-15 mg/ml) of undigested and digested yogurt samples for 24 hrs. Cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay (MTT I proliferation kit, Roche Diagnostics, UK). Sub-toxic concentrations of seaweed extract enriched yogurt and yogurt digestates were determined and used for subsequent experiments (Table 8). For the determination of cellular enzymatic activity, Caco-2 cells were supplemented with seaweed extract enriched yogurt and yogurt digestates (at concentrations outlined in Table 8) for 24 hrs. Following incubation, the CAT and SOD activities and GSH levels were determined. The Comet assay was used to access the potential DNA

protective effects of seaweed enriched yogurt and yogurt digestates in Caco-2 cells following 30 min incubation with 50 μM H_2O_2.

2.13. Statistical analysis

Statistical analysis for *in-vitro* antioxidant assays, surface colour, lipid oxidation, pH, microbiology, whey separation, and rheology measurements was by one-way ANOVA or repeated measures ANOVA followed by Dunnett's test or Tukey's test (Prism 4.0, GraphPad Inc, San Diego, CA, USA). Each experiment was carried out four times and results are presented as mean values ± the standard error of the mean (SEM). The level of statistical significance was $P < 0.05$.

ANOVA-Partial Least Squares Regression (APLSR) was used to process the mean data accumulated from the 26 test subjects during sensory analysis and shelf-life evaluation using instrumental methods. The X-matrix was designated as 0/1 for treatment and days with the Y-matrix designated as sensory and instrumental variables. The optimal number of components in the ASLSR models presented was determined to be 6 principal components. PC1 versus PC2 is presented as the other PCs did not yield additional information. In these models, assessor and session level effects were removed using level correction. The validated explained variance for the model constructed for yogurt samples was 69% and the calibrated variance was 73.19%. To derive significance indications for the relationships determined in the quantitative APLSR, regression coefficients were analyzed by jack-knifing which is based on cross-validation and stability plots (Martens & Martens, 1999, 2001). All analyses were performed using Unscrambler Software, version 9.8 (CAMO ASA, Trondheim, Norway).

3. Results and Discussion

3.1. Characterisation of seaweed extracts

The total phenol content of the seaweed extracts followed the order $FV_{60e} > AN_{80e} > AN_{100}$ ($P < 0.05$) (Table 1) and the TPC of these extracts were comparable to those of the ASE® extracts from Chapter 4. By contrast the FICA of the FV_{60e} was significantly lower than AN_{100}, AN_{80e}. These results highlight the complexity and range of compounds present in seaweed extracts which exhibit a variety of chemical activities. The DPPH radical scavenging activity was similar for all seaweed extracts and results were comparable to the DPPH radical scavenging activity of 60% methanol prepared seaweed extracts reported in Chapter 2. In Chapter 5 it was found that the maximum solubility of the seaweed extracts was 0.5% (w/w) and therefore in the current study seaweed extracts were added to milk at concentrations of 0.25% and 0.5% (w/w), prior to yogurt production.

3.2. Compositional analysis of seaweed extract enriched yogurt

The composition (moisture, fat, protein, ash and carbohydrate) of yoghurts containing seaweed extracts were similar to the control yoghurt and not affected by seaweed extract type or concentration (Table 2). Similar protein and ash concentrations in yogurt were reported by Sabeena Farvin et al. (2010a). Yoghurt containing seaweed extracts were comparable to the commercial natural yoghurt with respect to fat and ash contents. The commercial yoghurt contained higher carbohydrate and protein levels presumably due to the addition of milk protein as an ingredient in the yoghurt formulation.

3.3. Surface colour of yogurt

Colour is an important attribute in yogurt and according to the USDA natural unflavoured yogurt should be bright white to off-white in colour. Seaweed-derived extracts contain pigments such as tetrapyrroles (e.g. chlorophyll), carotenoids (e.g. β-carotene), and polyphenolic compounds (e.g. anthocyanins) (Schoefs, 2004) which may influence the colour of yogurt (Sanz et al., 2008). The addition of seaweed extracts did not significantly affect the surface lightness 'L*' values of yogurt (Table 3). Yogurts containing aqueous extracts from *A. nodosum* ((AN_{100} (0.25%) and AN_{100} (0.5%)) had significantly ($P < 0.05$) lower '-a*' values compared to other yogurts. Yellowness 'b*' values were significantly ($P < 0.05$) higher in yogurts

containing the extracts AN_{80e} (0.5%), FV_{60e} (0.25%) and FV_{60e} (0.5%) and . Similar results were reported in chapter 5 where milk samples containing AN_{80e} (0.25%), AN_{80e} (0.5%), FV_{60e} (0.25%), and FV_{60e} (0.5%) had higher "b*" yellowness values. The 'L*', '-a*' and 'b*' values of the seaweed extract enriched yogurts did not change significantly over the 28 day storage period.

Table 1

Total phenol content (TPC), 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH•) radical scavenging assay and ferrous-ion chelating assay (FICA) of seaweed extracts.

	AN_{100}[a]	AN_{80e}[b]	FV_{60e}[c]
TPC (mg gallic acid equivalent)	43.5 ± 5.5[b,c]	74.8 ± 3.5[a,c]	91.5 ± 6.5[a,b]
DPPH (% radical scavenging)	28.0 ± 3.0	26.5 ± 4.0	28.5 ± 5.0
FICA (%)	68.0 ± 8.0[c]	75.5 ± 7.5[c]	44.5 ± 6.0[a,b]

([a-c]) denotes a significant difference between mean values, repeated measures ANOVA followed by Tukey's post-test. Data without superscripts indicates no significant difference. n = 4 independent experiments.

Table 2
Compositional analysis of yogurt containing seaweed extracts.

	Composition (%)				
	Moisture	Fat	Protein	Ash	Carbohydrate
Control	88.0±0.2	2.7±0.2	3.0±0.1	0.6±0.1	5.7±0.1
AN_{100} (0.25%)	87.8±0.1	2.6±0.1	3.0±0.1	0.6±0.1	5.9±0.2
AN_{100} (0.5%)	87.7±0.1	2.8±0.2	3.1±0.2	0.7±0.1	5.7±0.5
AN_{80e} (0.25%)	88.1±0.3	2.8±0.2	2.8±0.1	0.7±0.1	5.6±0.4
AN_{80e} (0.5%)	87.7±0.1	2.7±0.2	3.1±0.2	0.7±0.1	5.8±0.3
FV_{60e} (0.25%)	88.2±0.3	2.4±0.1	3.0±0.1	0.7±0.1	5.8±0.5
FV_{60e} (0.5%)	87.9±0.3	2.5±0.1	3.1±0.2	0.7±0.1	5.7±0.4
Commercial yogurt	84.9±0.1	2.4±0.2	4.8±0.2	0.7±0.1	7.2±0.4

Mean values ± SEM. n = 3 independent experiments.

Table 3

Effect of seaweed extract addition on the surface lightness ("L*" value), greenness ("-a*") and yellowness ("b" value) of yogurt for up to 28 days at 4°C.

		Storage time at 4°C, days				
		1	7	14	21	28
Lightness "L*"	Control[a]	82.3±2.5	88.6±2.1	91.4±1.6	89.0±1.5	92.5±1.5
	AN_{100} (0.25%)[b]	84.4±0.8	84.9±0.5	85.6±1.9	87.4±1.3	89.0±0.3
	AN_{100} (0.5%)[c]	83.5±2.6	84.7±2.7	84.6±1.6	87.4±1.4	85.3±0.1
	AN_{80e} (0.25%)[d]	87.7±2.6	85.0±2.4	84.7±2.4	84.9±2.0	83.5±0.1
	AN_{80e} (0.5%)[e]	81.5±3.1	83.7±1.6	84.3±1.6	84.9±1.4	85.9±0.2
	FV_{60e} (0.25%)[f]	85.7±4.5	81.3±2.4	82.8±1.5	82.0±1.1	82.7±0.1
	FV_{60e} (0.5%)[g]	79.3±5.7	80.1±1.5	77.5±1.5	79.0±1.1	76.3±1.8
Greenness "-a*"	Control[h]	-4.0±0.1[i,j]	-4.0±0.2[i,j]	-3.8±0.1[i,j]	-4.0±0.2[i,j]	-3.9±0.1[i,j]
	AN_{100} (0.25%)[i]	-2.1±0.4[h,k-n]	-2.3±0.4[h,k-n]	-2.2±0.3[h,k-n]	-2.3±0.3[h,k-n]	-1.8±0.1[h,k-n]
	AN_{100} (0.5%)[j]	-1.7±0.3[h,k-n]	-1.7±0.4[h,k-n]	-1.3±0.4[h,k-n]	-1.7±0.4[h,k-n]	-1.0±0.1[h,k-n]
	AN_{80e} (0.25%)[k]	-3.8±0.1[i,j]	-3.7±0.3[i,j]	-3.8±0.2[i,j]	-3.8±0.[i,j]	-3.4±0.[i,j]
	AN_{80e} (0.5%)[l]	-4.0±0.2[i,j]	-4.1±0.2[i,j]	-4.1±0.1[i,j]	-4.2±0.3[i,j]	-3.7±0.1[i,j]
	FV_{60e} (0.25%)[m]	-4.6±0.3[i,j]	-4.3±0.3[i,j]	-4.1±0.1[i,j]	-4.2±0.2[i,j]	-3.8±0.2[i,j]
	FV_{60e} (0.5%)[n]	-4.6±0.5[i,j]	-4.3±0.3[i,j]	-4.3±0.1[i,j]	-4.2±0.3[i,j]	-3.7±0.2[i,j]
Yellowness "b*"	Control[o]	13.3±1.9[s-u]	12.5±1.2[s-u]	11.2±0.5[s-u]	12.9±1.2[s-u]	10.9±0.1[s-u]
	AN_{100} (0.25%)[p]	14.9±1.2[s-u]	15.3±0.7[s-u]	13.3±0.1[s-u]	14.5±0.5[s-u]	13.8±0.4[s-u]
	AN_{100} (0.5%)[q]	15.3±0.4[s-u]	15.9±0.7[s-u]	16.1±0.7[s-u]	16.6±0.4[s-u]	15.5±0.5[s-u]
	AN_{80e} (0.25%)[r]	15.4±0.3[s-u]	15.9±1.1[s-u]	16.1±1.4[s-u]	16.0±1.0[s-u]	17.5±0.2[s-u]
	AN_{80e} (0.5%)[s]	18.7±0.7[o-r,t-u]	19.6±0.8[o-r,t-u]	18.9±0.7[o-r,t-u]	19.3±0.5[o-r,t-u]	18.7±0.5[o-r,t-u]
	FV_{60e} (0.25%)[t]	26.1±1.3[o-s,u]	25.7±1.2[o-s,u]	25.6±0.5[o-s,u]	25.8±1.2[o-s,u]	23.5±0.5[o-s,u]
	FV_{60e} (0.5%)[u]	30.8±1.3[o-t]	29.6±1.1[o-t]	29.9±0.4[o-t]	29.7±1.3[o-t]	26.9±0.3[o-t]

([a-g]) denotes a significant difference between mean values of Lightness "L*" values. ([h-n]) denotes a significant difference between mean values of Greenness "-a*" values. ([o-u]) denotes a significant difference between mean values of Yellowness "b*" values. Data without superscripts indicates no significant difference. One-way ANOVA followed by Tukey's post-test. n = 4 independent experiments.

3.4. Lipid oxidation in yogurt

Lipid oxidation can result in the formation of off-flavours and is another factor governing consumer acceptability of foods. In general, lipid oxidation does not occur in yogurt due to the low pH, low storage temperature, and the presence of naturally occurring antioxidant milk proteins (Serra et al., 2008). In addition, fermentation of milk by lactic acid bacteria releases a large number of peptides and amino acids into yogurt with antioxidant activity (Sabeena Farvin et al., 2010b). The fortification of yogurt with iron has been found to significantly increase the rate of lipid oxidation in the yogurt (Hekmat & McMahon, 1997). Seaweed extracts such as those used in the present study may contain a range of minerals and metals such as iron (Robledo & Pelegrin, 1997) which may initiate oxidation in yogurts. Lipid oxidation increased in each of the yogurt samples over the 28 day storage period and by day 28 the oxidation in the control sample had increased to 0.22 mg MDA/kg yogurt (Table 4). Yogurts containing AN_{80e} (0.5%) and FV_{60e} (0.5%) exhibited significantly ($P < 0.05$) lower levels of lipid oxidation compared to the control and other yogurts, which may be attributed to various antioxidant compounds present in the extracts suggesting a possible future food preservative use for these extracts. In chapter 5 FV_{60e} (0.25%) significantly reduced the level of lipid oxidation in milk. Similar findings were reported in previous studies where seaweed extracts reduced lipid oxidation in fish oil (Athukorala et al., 2003; Senevirathne et al., 2006), vegetable oil (Athukorala et al., 2003) and fish meat (Wang et al., 2010).

3.5. pH and microbiological analysis of yogurt

Starter culture bacteria, such as *S. thermophilus* and *L. bulgaricus*, are used to produce acid for the pH-dependent formation of yogurt. Low pH and the presence of starter cultures inhibit the growth of many spoilage and pathogenic bacteria ensuring a long shelf life (Ledenbach, & Marshall, 2009) and also impact on the flavour of yogurt. Seaweed extracts have exhibited potent antimicrobial effects against a range of bacteria including species from the genus *Lactobacillus* and *Streptococcus* (Sumathi & Krishnaveni, 2012). In the present study, the addition of seaweed extracts did not significantly affect the pH (Figure 1) or microbiological content (Table 5) of the resulting yogurts over the 28 day storage period. The pH of yogurts on day 1 ranged from 3.99 in AN_{100} (0.25%) to 4.09 in FV_{60e} (0.5%). By day 28 the

Table 4
Lipid oxidation in seaweed-enriched yogurt over 28 days at 4°C.

	Storage time at 4°C, days				
	1	7	14	21	28
Control[a]	0.12±0.06	0.16±0.03	0.21±0.02	0.23±0.02	0.22±0.01[e,g]
AN_{100} (0.25%)[b]	0.09±0.05	0.13±0.03	0.14±0.02	0.16±0.01	0.20±0.01[e,g]
AN_{100} (0.5%)[c]	0.10±0.05	0.17±0.02	0.15±0.03	0.18±0.02	0.18±0.03[e,g]
AN_{80e} (0.25%)[d]	0.09±0.05	0.14±0.04	0.12±0.03	0.17±0.02	0.18±0.01[e,g]
AN_{80e} (0.5%)[e]	0.09±0.04	0.11±0.04	0.11±0.03	0.12±0.03	0.16 ±0.01[a-f]
FV_{60e} (0.25%)[f]	0.10±0.05	0.12±0.03	0.10±0.04	0.14±0.02	0.19±0.02[e,g]
FV_{60e} (0.5%)[g]	0.11±0.06	0.10±0.03	0.09±0.03	0.14±0.02	0.15 ±0.01[a-d,f]

([a-g]) denotes a significant difference between mean values. Data without superscripts indicates no significant difference. Repeated measures ANOVA followed by Tukey's post-test. n= 4 independent experiments.

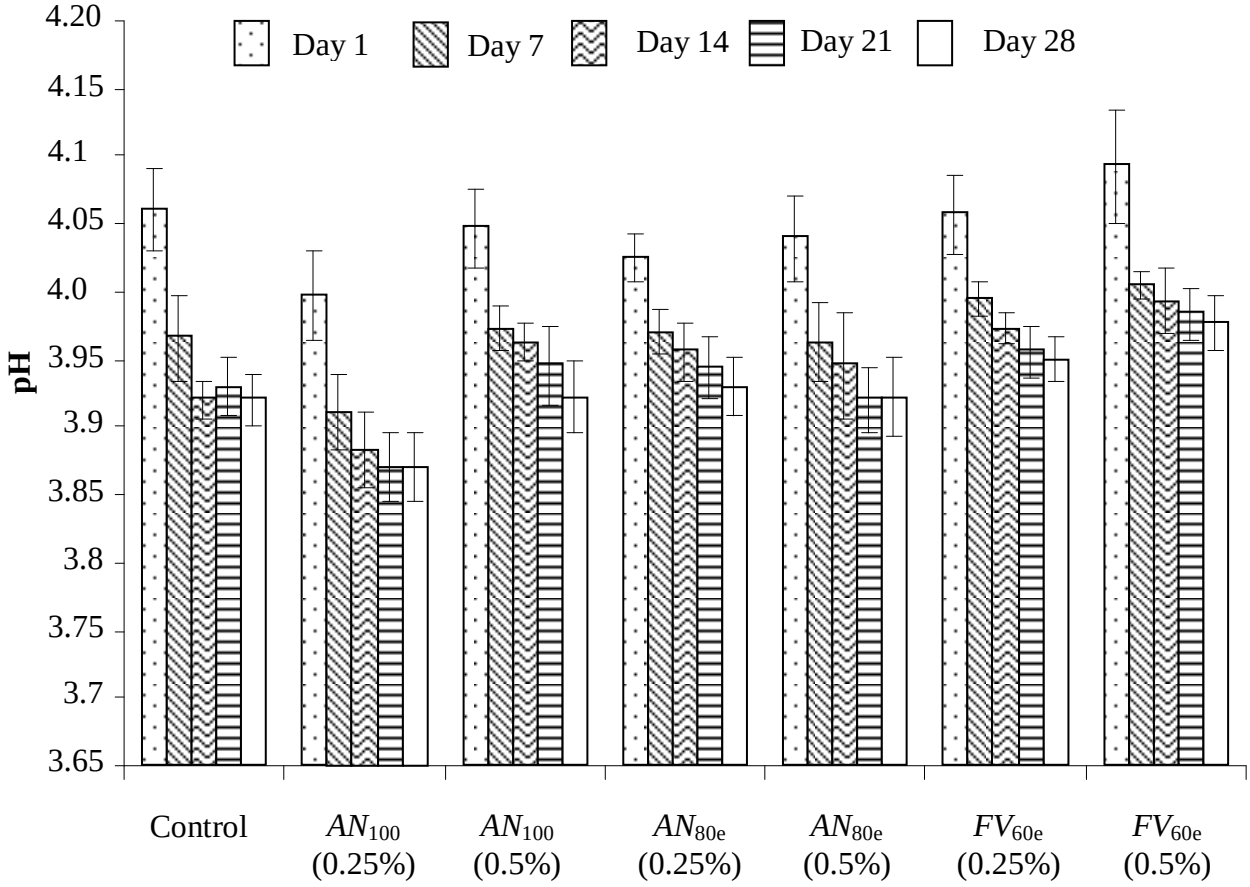


Figure 1. pH change in yogurt samples over 28 days storage at 4°C. Data are the means of four independent experiments, with standard errors represented by vertical bars. Statistical analysis was by one-way ANOVA followed by the Tukey's test. No significant differences between treatment means on each measurement day ($P > 0.05$).

Table 5
Effect of seaweed extracts addition on the microbial content of yogurt over 28 days at 4°C.

		Storage time at 4°C, days				
		1	7	14	21	28
Control	*Streptococcus thermophilus* (log_{10}CFU/g)	9.2±0.8	9.7±1.3	9.1±0.9	9.0±0.4	9.5±0.3
AN_{100} (0.25%)		8.7±0.9	9.7±0.7	8.9±0.9	9.1±0.5	9.7±1.6
AN_{100} (0.5%)		9.7±1.1	8.7±1.0	9.9±1.0	9.3±0.1	9.9±0.9
AN_{80e} (0.25%)		9.9±0.4	9.1±0.9	8.7±0.8	9.0±0.7	9.8±1.4
AN_{80e} (0.5%)		8.4±0.8	8.9±0.9	9.4±0.4	8.7±0.9	9.4±0.8
FV_{60e} (0.25%)		8.5±0.7	8.9±0.9	9.5±0.9	8.9±0.8	9.5±1.1
FV_{60e} (0.5%)		9.5±1.9	9.0±1.1	8.5±1.6	9.1±1.0	9.5±0.6
Control	*Lactobacillus* (log_{10}CFU/g)	6.9±0.9	6.5±0.8	7.5±0.7	7.0±1.2	7.1±0.9
AN_{100} (0.25%)		7.1±1.1	6.5±0.2	7.2±0.9	7.0±0.9	6.5±1.0
AN_{100} (0.5%)		7.5±0.9	7.1±1.3	7.4±1.1	7.2±0.9	7.1±0.8
AN_{80e} (0.25%)		7.2±0.9	7.0±1.7	7.1±0.9	6.5±0.9	7.5±0.9
AN_{80e} (0.5%)		6.5±1.3	6.9±0.9	7.0±0.7	7.2±0.6	7.9±1.7
FV_{60e} (0.25%)		7.5±0.7	7.1±1.1	6.7±0.9	7.0±0.7	6.5±0.9
FV_{60e} (0.5%)		7.1±0.8	6.9±0.7	7.5±0.7	6.9±1.2	7.5±0.9

Data without superscripts indicates no significant difference. Statistical analysis was by one-way ANOVA followed by the Tukey's test. n = 4 independent experiments.

pH ranged from 3.87 in AN_{100} (0.25%) to 3.98 in FV_{60e} (0.5%) (Figure 1). Growth of *S. thermophilus* ranged from 8.4 in AN_{80e} (0.5%) on day 1 to 9.9 in AN_{100} (0.5%) on day 28 while *L. bulgaricus* ranged from 6.5 in AN_{80e} (0.5%) on day 1 to 7.9 in AN_{80e} (0.5%) on day 28. Similar growth rates were observed in banana yogurt (Çakmakçi et al., 2012). In previously reported studies antioxidants (vitamin C, vitamin E and β-carotene) (Brignac & Aryana, 2012) and Maritime Pine-derived pycnogenol (Ruggeri et al., 2008) did not affect the growth of the starter cultures or the pH of yogurts during storage. In chapter 5 the addition of seaweed extracts did not affect the pH or microbiology of milk.

3.6. Whey separation in yogurt

Whey separation, a phenomenon normally caused by spontaneous gel contraction (syneresis), is a fault that occurs when whey is expelled from the gel network of yogurt resulting in the pooling of whey on the yogurt surface (Lee & Lucey, 2010). Whey separation is unappealing to consumers as it has an appearance of spoilage and can be reduced through the use of protein or stabilisers such as pectin and gelatin. The rate of whey separation can be influenced by the addition of certain antioxidant materials such as carrot-derived antioxidants (Salwa et al., 2004). In the present study, whey separation was similar for all yogurt samples over the 28 day storage period (Figure 2). Similarly, Brignac & Aryana (2012) found that the addition of vitamin C, vitamin E and β-carotene to yogurt did not influence whey separation.

3.7. Rheological analysis of yogurt

Rheological attributes are important in yogurt (Lovely & Meullenet, 2009). The shear modulus (*G*) measures the tendency of yogurt to deform when acted on by opposing forces which relates to the viscosity of yogurt. The modulus peaked in AN_{100} (0.25%), AN_{100} (0.5%) and FV_{60e} (0.25%) on day 21 and by day 28 had significantly decreased in FV_{60e} (0.25%). The modulus of AN_{80e} (0.25%), AN_{80e} (0.5%) and FV_{60e} (0.5%) peaked on day 21. The modulus of FV_{60e} (0.5%) subsequently decreased on following days. The control yogurt exhibited significantly higher modulus on day 28 compared to the other samples apart from AN_{100} (0.5%) and AN_{80e} (0.25%). By day 28 the yogurt containing FV_{60e} (0.5%) had the lowest modulus of all yogurts (Table 6). The modulus of the yogurts is considerably lower than that reported by Lee & Lucey (2004) which may be due to

the lower starter culture inoculation rates (0.1%) used in the present study. In general the modulus of seaweed extract-enriched yogurts was lower than that of the control yogurt.

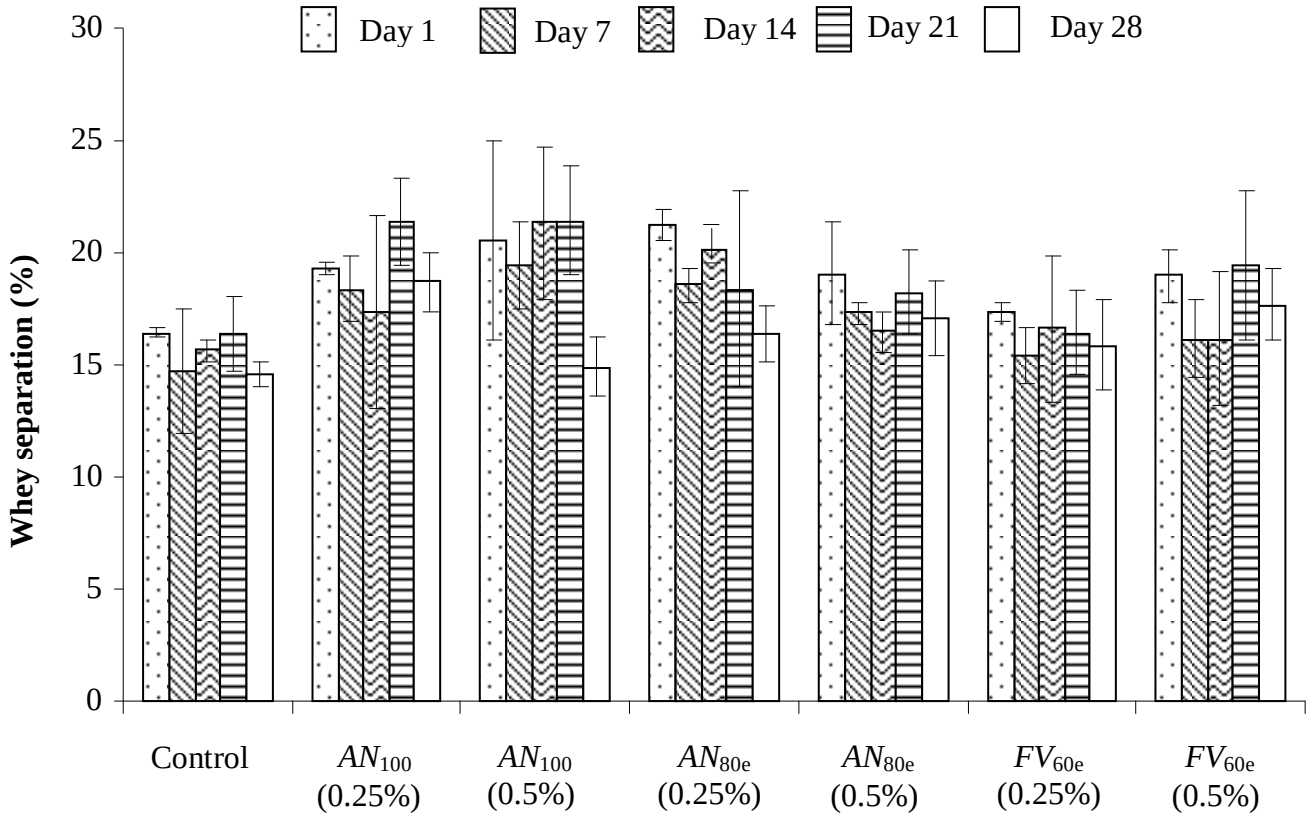


Figure 2. Measure of whey separation (%) in yogurt samples over 28 days storage at 4°C. Data are the means of four independent experiments, with standard errors represented by vertical bars. Statistical analysis was by one-way ANOVA followed by the Tukey's test. No significant differences between treatment means on each measurement day ($P > 0.05$).

Table 6

The shear modulus of seaweed extract enriched yogurt over 28 days at 4°C.

	Storage time at 4°C, days				
	1	7	14	21	28
Control[a]	55±6.0	59.5±9.5	88.5±17.0	83.5±9.5	98.5±16.5[b,e-g]
AN_{100} (0.25%)[b]	43.5±2.0	43.5±3.5	50.5±5.5	48.5±5.5	50.5±1.5[a,c,d,g]
AN_{100} (0.5%)[c]	54.5±7.5	58.5±14.5	69.5±11.5	67.5±6.5	64.5±5.5[b,e-g]
AN_{80e} (0.25%)[d]	45.5±9.0	51.5±12.5	58.5±13.5	60.5±6.5	61.5±7.5[b,e-g]
AN_{80e} (0.5%)[e]	39.5±7.5	51.5±9.59	46.5±4.5	49.5±7.5	43.5±1.5[a,c,d,g]
FV_{60e} (0.25%)[f]	44.5±7.0	40.0±3.5	60.5±10.5	57.5±6.5	47.5±2.5[a,c,d,g]
FV_{60e} (0.5%)[g]	37.5±7.5	47.0±13.5	55.5±9.5	56.5±7.5	33.5±3.5 [a-f]

([a-g]) denotes a significant difference between mean values. Data without superscripts indicates no significant difference. Repeated measures ANOVA followed by Tukey's post-test. n = 4 independent experiments.

3.8. Sensory evaluation of yogurt

Sensory evaluation, instrumental and chemical analysis data is presented in the APLSR plot (Figure 3) with the corresponding ANOVA values in Table 7. In general, positive and negative correlations indicate like and dislike of selected attributes, respectively. The control yogurt ($P < 0.001$) and yogurts containing AN_{100} (0.25%) ($P < 0.001$), AN_{100} (0.5%) ($P < 0.05$) and FV_{60e} (E) (0.5%) ($P < 0.001$) were significantly positively correlated with the colour descriptor. Yogurt containing AN_{80e} (0.5%) and FV_{60e} (0.5%) exhibited a negative correlation to colour (Table 7 and Figure 3) probably due the higher instrumental "b*" yellowness values (Table 3) observed in these yogurts.

The control yogurt ($P < 0.001$) and AN_{100} (0.25%) ($P < 0.01$) and AN_{100} (0.5%) ($P < 0.001$) containing yogurts were positively correlated to texture. Yogurts containing AN_{80e} (0.25%) ($P < 0.01$) and AN_{80e} (0.5%) ($P < 0.001$) were found to be negatively correlated to odour. The flavour of the control yogurt ($P < 0.001$) and yogurts containing AN_{100} (0.25%) ($P < 0.05$) and AN_{100} (0.5%) ($P < 0.01$) were the most preferred by sensory panellists. Yogurts containing AN_{80e} (0.25%), AN_{80e} (0.5%) and FV_{60e} (0.5%) ($P < 0.01$) were negatively correlated to flavour and it is postulated that the undesirable colour of these yogurts may have adversely biased the flavour-liking scores of sensory panellists. In traditional foods like yogurt unexpected colours can negatively affect flavour perception (Sanz et al., 2008). The yogurt containing AN_{80e} (0.5%) ($P < 0.001$) exhibited a positive correlation to off-flavour however the other yogurts were not affected by the addition of seaweed extracts. The control yogurt ($P < 0.001$) and yogurts containing AN_{100} (0.25%) ($P < 0.01$) and AN_{100} (0.5%) ($P < 0.01$) were positively correlated to overall acceptability (Table 7). In chapter 5 the control milk and AN_{100} (0.5%) containing milk were positively correlated to overall acceptability. Yogurts containing AN_{80e} ($P < 0.01$) and FV_{60e} ($P < 0.01$) were negatively correlated to overall acceptability indicating reduced acceptability by sensory panellists. Overall the data suggested that colour, flavour and texture were the three most important parameters governing the overall acceptability of yogurt containing seaweed extracts.

The sensory properties of yogurt as a function of time were also examined (Table 7 and Figure 3). Odour, flavour, and overall acceptability were positively correlated to day 1, 7 and 14 and negatively correlated to day 21 and 28. These results indicated that sensory attributes of yogurt decreased after day 14 of storage. Similarly

Table 7
P-values of regression coefficients as derived by jack-knife uncertainty testing.

	Yogurt							Storage time, at 4°C, days				
	Control	AN_{100} (0.25%)	AN_{100} (0.5%)	AN_{80e} (0.25%)	AN_{80e} (0.5%)	FV_{60e} (0.25%)	FV_{60e} (0.5%)	1	7	14	21	28
Sensory Attribute												
Colour	0.001***	0.001***	0.05*	-0.506ns	-0.01**	0.001***	-0.001***	0.83ns	0.575ns	0.01**	-0.01**	-0.55ns
Texture	0.001***	0.05*	0.001***	-0.103ns	-0.247ns	0.001***	-0.05*	0.212ns	0.226ns	0.174ns	-0.396ns	-0.693ns
Odour	0.001***	0.054ns	0.16ns	-0.01**	-0.001***	0.229ns	-0.107ns	0.001***	0.05*	0.001***	-0.001***	-0.001***
Flavour	0.001***	0.05*	0.01**	-0.05*	-0.001***	0.21ns	-0.01**	0.01**	0.01**	0.05*	-0.05*	-0.001***
Off-flavour	-0.001***	-0.001***	-0.084ns	0.21ns	0.001***	-0.73ns	0.12ns	-0.001***	-0.05*	-0.01**	0.001***	0.375ns
Thickness	0.001***	0.77ns	0.001***	-0.18ns	-0.83ns	0.95ns	-0.41ns	0.001***	0.23ns	0.001***	-0.01*	-0.001***
Overall acceptability	0.001***	0.01**	0.01**	-0.061ns	-0.01**	0.32ns	-0.01**	0.001***	0.01**	0.01**	-0.001***	-0.001***
Instrumental and chemical analysis												
pH	-0.39ns	-0.001***	-0.144ns	-0.92ns	0.001***	0.001***	0.001***	0.001***	-0.14ns	-0.001***	-0.001***	-0.001***
Tbars	0.001***	0.59ns	0.001***	0.77ns	-0.001***	-0.063ns	-0.001***	-0.001***	0.68ns	0.001***	0.001***	0.001***
Syneresis	-0.001***	-0.01**	-0.001***	-0.05*	0.96ns	0.001***	0.19ns	0.001***	-0.057ns	-0.86ns	-0.001***	-0.001***
L	0.001***	0.001***	0.2ns	0.05*	-0.15ns	-0.001***	-0.001***	-0.13ns	0.23ns	0.964ns	0.05*	0.056ns
a	0.001***	0.001***	0.001***	0.24ns	-0.001***	-0.001***	-0.001***	-0.05*	0.67ns	0.63ns	0.01**	0.001***
b	-0.001***	-0.001***	-0.001***	-0.001***	0.83ns	0.001***	0.001***	0.34ns	-0.79ns	-0.48ns	-0.18ns	-0.068ns
DPPH	-0.001***	-0.001***	-0.54ns	-0.001***	0.4ns	0.001***	0.001***	0.62ns	-0.19ns	-0.76ns	-0.001***	-0.01**
Rheology	-0.001***	-0.001***	-0.22ns	-0.47ns	0.001***	0.001***	0.43ns	0.068ns	-0.01**	-0.555ns	-0.01*	-0.368ns

Significance of regression coefficients; ns, not significant, *95% significance, $P < 0.05$, **99% significance, $P < 0.01$, ***99.9% significance, $P < 0.001$. n = 4 independent experiments.

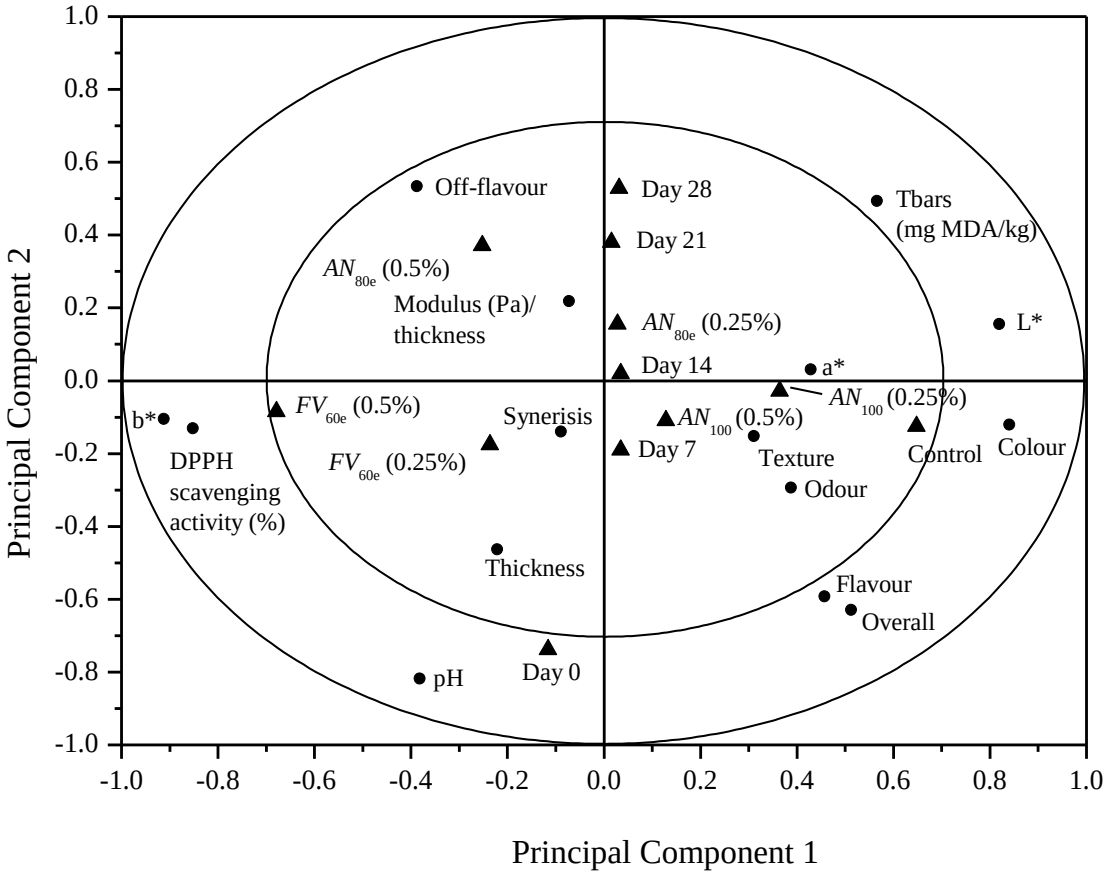


Figure 3. APLSR for the various seaweed-enriched yogurts. ▲=Yogurt sample, ●=sensory descriptor, chemical and instrumental variables. The concentric circles represent 100% and 50% explained variance, respectively.

Çakmakçi et al. (2012) reported decreased sensory quality of banana yogurt after 7 days of storage due to the development of acidity in the yogurts.

Negative sensory associations with some of the seaweed extract enriched yogurts examined in the present study could potentially be addressed using food colorants, flavourings or by micro-encapsulation of the extracts in order to mask undesirable colour and flavours associated with seaweed extracts (Petrotos et al., 2012).

3.9. Stability of seaweed extracts in yogurt

Previous studies have reported that the antioxidant activity of yogurts enriched with tea polyphenols (Najgebauer-Lejko et al., 2011) and vitamins C and A (Ilic & Ashoor, 1988) decreased as a function of storage time. The DPPH radical scavenging activity of all yogurts was similar over the 28 day storage period indicating stability of the seaweed extracts in yogurt (Figure 4). Yogurts containing FV_{60e} (0.25%) and FV_{60e} (0.5%) exhibited the highest DPPH radical scavenging activity similar to that of Trolox (0.04 µM) standard. These findings indicated that the seaweed extracts are stable antioxidant ingredients within a fermented dairy product such as yogurt. In chapter 5 seaweed extracts were also stable in milk as a function of storage time.

3.10. *In-vitro* and cellular antioxidant activity of yogurts and digestates

The antioxidant potential of the seaweed enriched yogurts was determined before and after an *in-vitro* digestion procedure by measuring the DPPH radical scavenging activity and the ferrous iron chelating ability. Antioxidant effects were further investigated by measuring cellular antioxidant activity and protection against oxidant-induced DNA damage in Caco-2 cells supplemented with the seaweed enriched yogurts or yogurt digestates. The yogurt containing FV_{60e} (0.5%) was found to have a significantly ($P < 0.05$) higher radical scavenging activity than the control before digestion. In addition DPPH radical scavenging activity only occurred in FV_{60e} (0.5%) digestate following the *in-vitro* digestion procedure (Figure 5). DPPH scavenging activity has also been shown to decrease following *in-vitro* digestion in rosemary (*Rosmarinus officinalis*), wild-grown caper (*Capparis spinosa* L.) and sea fennel (*Crithmum maritimum* L.) (Soler-Rivas et al., 2010; Siracusa et al., 2011) Antioxidant compounds such as polyphenols may be degraded during digestion due to alterations in the pH and enzymatic hydrolysis (Stalmach et al.,

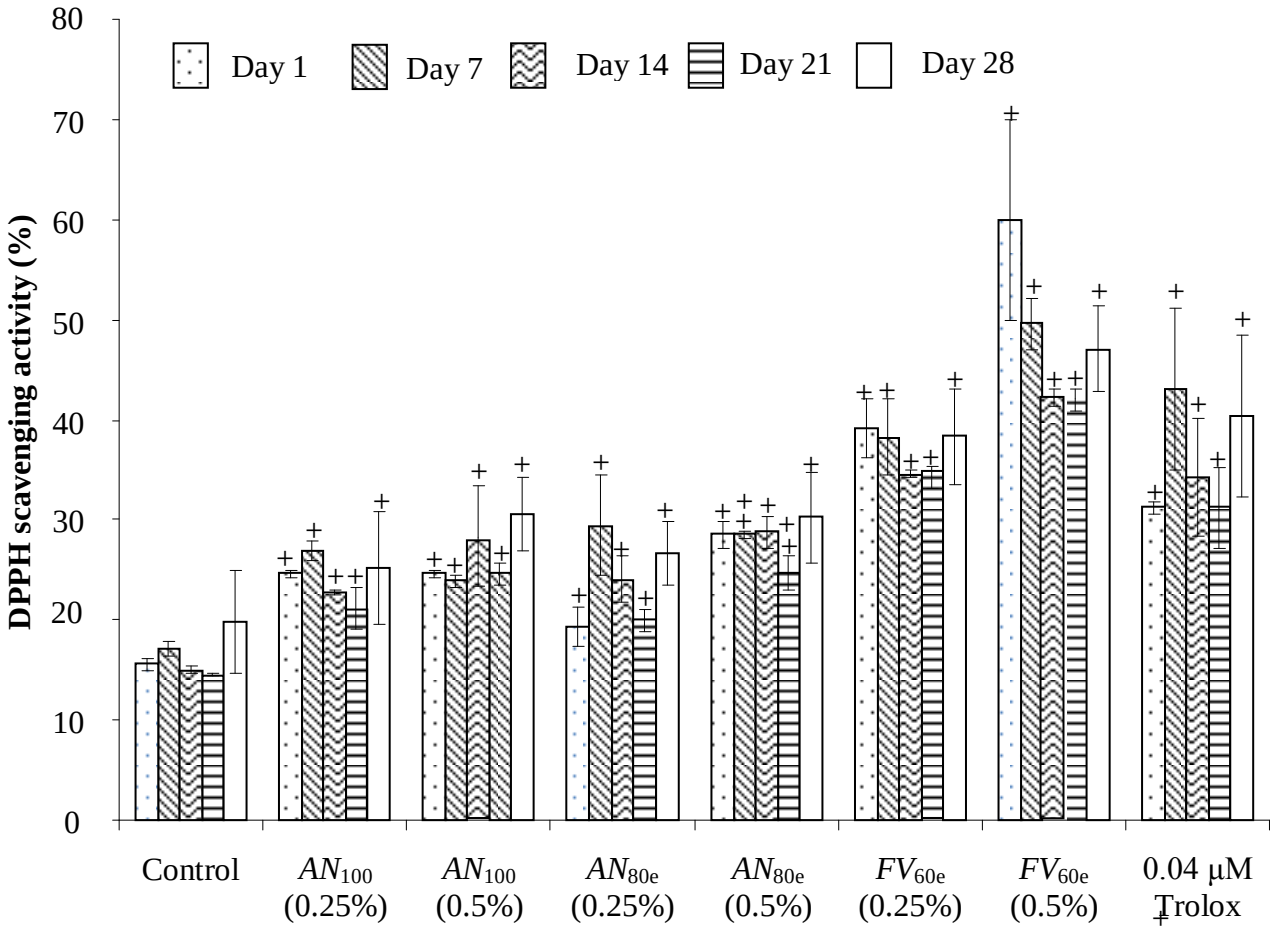


Figure 4. The DPPH-scavenging activity stability of yogurt samples over 28 days storage at 4°C. Data are the means of four independent experiments, with standard errors represented by vertical bars. $^{+}$ denotes significantly higher ($P < 0.05$) DPPH radical scavenging activity, on each measurement day, between seaweed extract enriched yoghurt samples and the corresponding controls.

2012). By contrast, in chapter 5 the DPPH-scavenging activity of seaweed-enriched milk samples was stable during digestion. This indicates that seaweed extracts prepared from *Ascophyllum nodosum* were less stable in the fermented milk than in non-fermented milk.

Dairy products such as yogurt can efficiently bind iron due to the presence of proteins such as casein, lactoferrin, whey albumin, transferrin and other milk proteins (Gallaher et al., 2005; Bullen et al., 2007). Prior to digestion, the yogurt containing FV_{60e} exhibited the highest FICA ($P < 0.05$) while the other yogurt samples had similar FICA. The FICA of all yogurt samples increased following digestion and there was no significant difference between the different digestates (Figure 6). A similar increase occurred in milk following digestion (Chapter 5). The increased FICA exhibited in the yogurt digestates may be due to the presence of milk peptides and iron-chelating components such as polyphenols from seaweed extracts released during the *in-vitro* digestion procedure (Hurrell et al., 2006).

Non toxic concentrations of the digested and undigested yogurt samples were determined using the MTT assay and yogurts were added to Caco-2 cells at 10 mg/ml media (0.05 mg seaweed extract) and digestates at 0.66 mg/ml media (0.0033 mg seaweed extract). Cellular antioxidant effects were examined by measuring the SOD and CAT activities and the GSH content in Caco-2 cells exposed to the yogurt and yogurt digestates for 24 hrs. It was found that the addition of seaweed-enriched yogurt or digestates did not significantly alter the antioxidant status in Caco-2 cells (Table 9). The DNA protective effects of the seaweed extract enriched yogurts and yogurt digestates against H_2O_2 induced DNA damage in Caco-2 cells was determined using the comet assay. Caco-2 cells were preincubated with the yogurt or digestates for 24 hrs and DNA damage was induced by the addition of 50 µM H_2O_2 which increased DNA damage from a control level of 8.5% to 56% (Figure 7). None of the yogurt or digestate samples protected against DNA damage in Caco-2 cells under the conditions employed in the present study.

It has been reported previously that crude extracts and isolated compounds from seaweed enhanced antioxidant enzyme activity and protected against oxidant induced DNA damage in cells (Kang et al., 2005; O'Sullivan et al., 2011) however, at comparable seaweed extract concentrations, the seaweed supplemented yogurts did not demonstrate antioxidant activity in Caco-2 cells. Previous studies have found

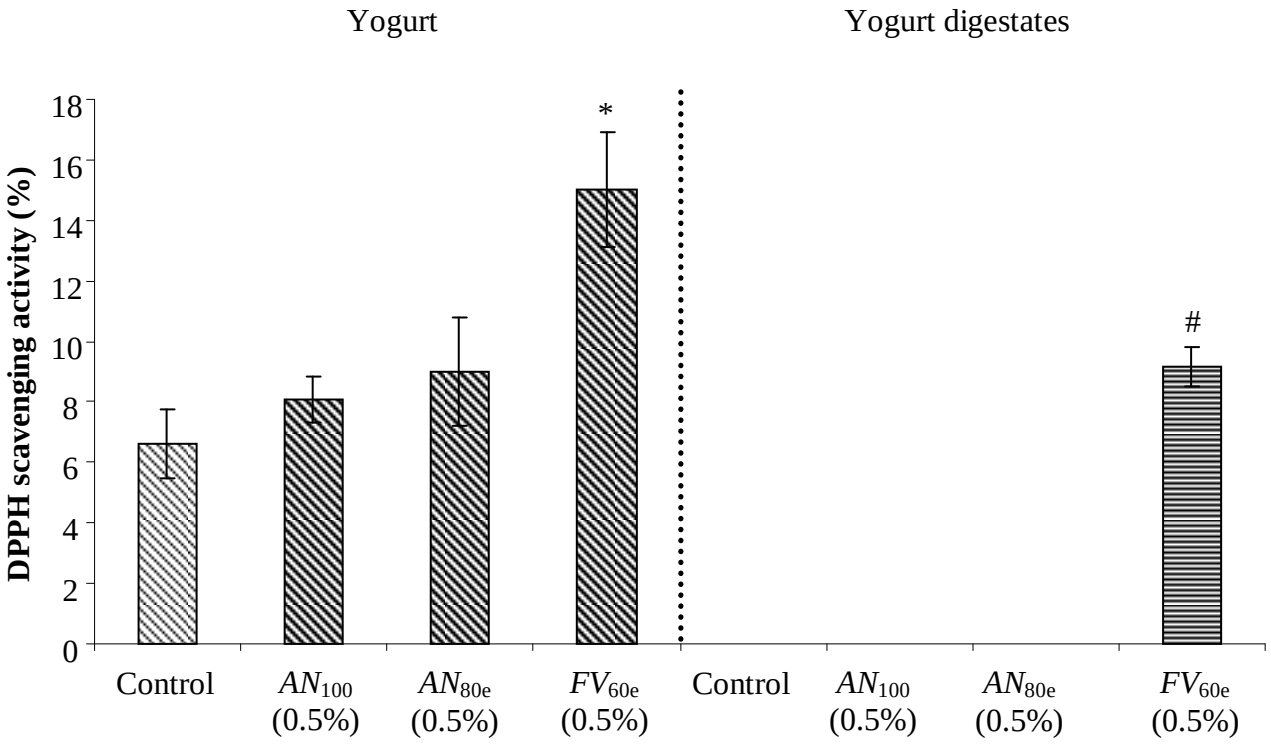


Figure 5. The DPPH-scavenging activity of yogurt samples before and after an *in-vitro* digestion procedure. Data are the means of four independent experiments, with standard errors represented by vertical bars. * denotes significantly higher DPPH scavenging activity values compared to other yogurt samples as determined using repeated measures ANOVA followed by Tukey's post-test. #denotes significantly different DPPH scavenging activity values compared to other digested yogurt samples

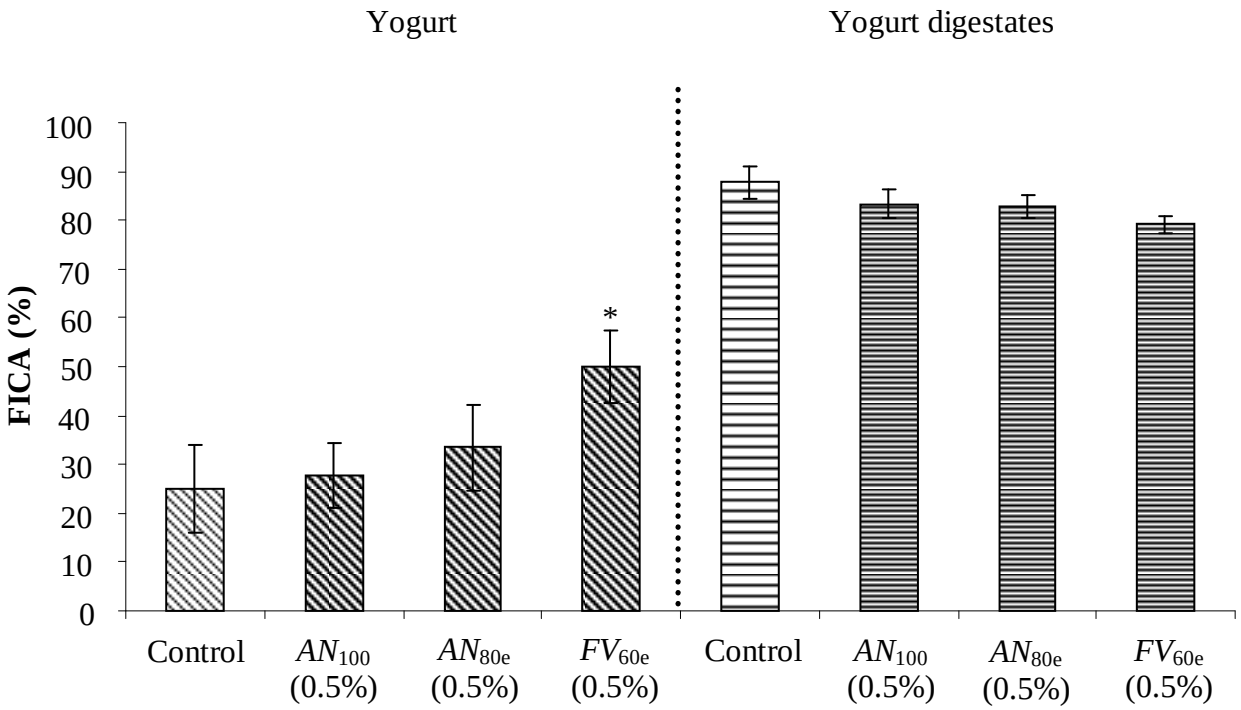


Figure 6. The ferrous-ion chelating activity (FICA) of yogurt samples before and after an *in-vitro* digestion procedure. Data are the means of four independent experiments, with standard errors represented by vertical bars. * denotes significantly different FICA values compared to other samples as determined using repeated measures ANOVA followed by Tukey's post-test.

Table 8

Viability of Caco-2 cells following addition of increasing levels (mg/ml) of seaweed-enriched yogurts

	Weight of yogurt added, mg			
	0	5	10	15
Undigested samples		% viability		
Control	100.0	103.0	93.5	83.2
AN_{100} (0.5%)	100.0	96.4	95.4	74.3
AN_{80e} (0.5%)	100.0	106.8	97.0	81.2
FV_{60e} (0.5%)	100.0	111.1	107.0	89.7
	Weight of yogurt added, mg			
	0	0.3	0.67	1.0
Digested samples		% viability		
Control	99.9	89.2	90.0	59.2
AN_{100} (0.5%)	100.0	98.6	85.9	61.7
AN_{80e} (0.5%)	100.0	101.8	91.6	75.8
FV_{60e} (0.5%)	100.0	105.4	92.2	73.8

Mean values ± SEM. n = 3 independent experiments.

Table 9

The effect of seaweed-enriched yogurt and yogurt digestates on the antioxidant status of Caco-2 cells.

	SOD activity (% Control)	CAT activity (% Control)	GSH content (% Control)
Undigested samples			
AN_{100} (0.5%)	105.5 ± 7.0	99.5 ± 6.5	100 ± 8.5
AN_{80e} (0.5%)	95.0 ± 6.0	97.0 ± 2.0	107.0 ± 10.0
FV_{60e} (0.5%)	96.5 ± 5.0	97.5 ± 8.0	100.5 ± 7.5
Digested samples			
AN_{100} (0.5%)	98.5 ± 7.5	97.5 ± 1.5	105.5 ± 1.5
AN_{80e} (0.5%)	95.0 ± 5.5	90.5 ± 6.5	99.5 ± 5.5
FV_{60e} (0.5%)	95.5 ± 9.0	95.5 ± 7.5	103.5 ± 10.0

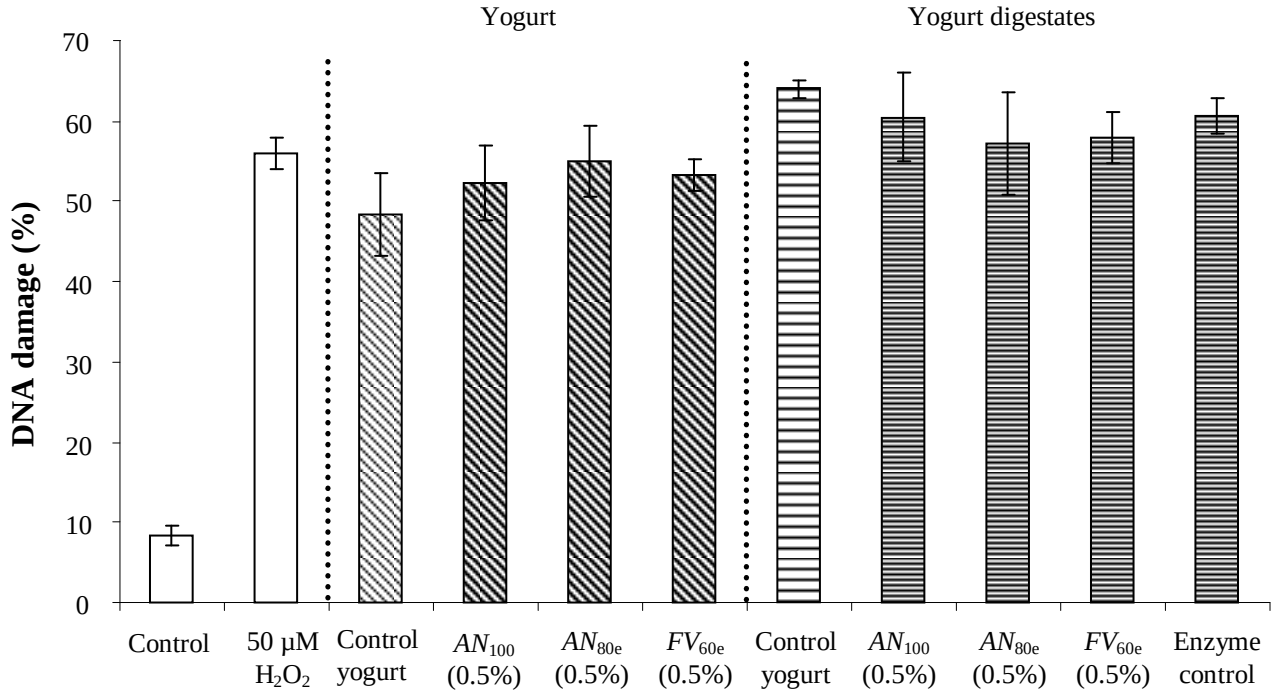


Figure 7. DNA damage in Caco-2 cells following pre-treatment with or without seaweed-enriched yogurt or yogurt digestates for 24 hrs then exposed to 50 µM H_2O_2 for 30 min at 37°C. DNA damage was assessed by the comet assay (explained earlier) in which % tail DNA was measured. Data are the means of four independent experiments, with standard errors represented by vertical bars. Statistical analysis was by repeated measures ANOVA followed by the Dunnett's test.

that, in the presence of milk proteins, the DPPH scavenging and antioxidant activity of a number of polyphenol compounds was reduced due to the formation of polyphenol-milk protein complexes (Xiao et al., 2011; Lorenz et al., 2007; Ryan & Petit, 2010). Furthermore, heat denatured proteins, such as those found in yogurt, are more likely to form polyphenol-milk protein complexes due to the increased exposure of polyphenol binding sites (Siebert et al., 1996). This may explain the lack of cellular antioxidant activity by the undigested yogurt samples. The lack of cellular antioxidant activity in digested yogurt samples may be due to the pH-mediated modification of compounds present in the seaweed extracts which occurred during the *in-vitro* digestion procedure.

4. Conclusions

Seaweed extract enriched yogurts were manufactured with 0.25 and 0.5% seaweed extracts however a number of seaweed extracts altered the characteristics of the resulting yogurts. In particular, yogurts containing FV_{60e} (0.25%), FV_{60e} (0.5%) and AN_{80e} (0.5%) had higher yellowness 'b*' values than other yogurt samples. From a quality perspective, yogurts containing AN_{80e} (0.5%) and FV_{60e} (0.5%) exhibited lower levels of lipid oxidation which may suggest a possible food preservative role for these extracts. Parameters such as pH, microbiology and whey separation were not affected by the addition of seaweed extracts to yogurt. This suggests that seaweed extracts may be added to yogurt without negatively affecting shelf-life characteristics. Sensory analysis indicated that colour, flavour and texture were the three most important parameters governing the overall acceptability of seaweed-enriched yogurt. Yogurts containing AN_{100} (0.25%) and AN_{100} (0.5%) were the most acceptable yogurts from a sensory perspective. Results from the *in-vitro* and cellular antioxidant assay indicate that the ferrous-ion chelating activity of yogurts is stable after digestion, however the DPPH-scavenging activity is not stable. In addition, the seaweed extract-enriched yogurts did not exhibit cellular antioxidant activity indicating reduced biological activity of extracts when added to yogurt. Further research is needed to evaluate the potential of additional seaweed derived ingredients as functional components in fermented dairy products.

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CHAPTER 7

Assessment of the antioxidant potential of extracts of Chlorella, Spirulina and Kelp food supplements in Caco-2 cells.

Abstract

The antioxidant effects of commercially sourced algal nutritional supplements *Ascophyllum nodosum*, *Chlorella vulgaris* and *Spirulina platensis* were assessed. Extracts of the supplements were prepared using 60% aqueous methanol and yields from *C. vulgaris*, *S. platensis* and *A. nodosum* were 2%, 8.5% and 7%, respectively. The antioxidant potential of the extracts was assessed by measuring the total phenol content (TPC), ferric reducing antioxidant power (FRAP) and ability to prevent β-carotene bleaching (β-CB). The effect of the extracts on the antioxidant status in Caco-2 cells was measured and the comet assay was used to determine the protective effects of the extracts against hydrogen peroxide (H_2O_2) and *tert*-Butyl hydroperoxide (*tert*-BOOH)-induced DNA damage. *A. nodosum* exhibited the highest TPC and FRAP activity. *C. vulgaris* and *S. platensis* were the most effective at preventing β-CB. *C. vulgaris* and *S. platensis* extracts significantly increased the glutathione (GSH) content in Caco-2 cells, while the *A. nodosum* extract significantly decreased the GSH content. There was no significant alteration in the activity of the antioxidant enzymes, catalase and superoxide dismutase, following incubation with the extracts. None of the extracts protected against H_2O_2-induced DNA damage however, *S. platensis* protected against *tert*-BOOH -induced DNA damage. In conclusion, while each of the extracts demonstrated antioxidant potential by TPC, FRAP and β-CB however, only limited antioxidant effects were observed in Caco-2 cells.

1. Introduction

Seaweed consumption in western countries is low in comparison to countries such as Japan, China and Korea where seaweed based products can form a large proportion of the daily staple diet. Recent studies suggest that seaweed derived antioxidants may reduce the risk of a range of human diseases including heart disease and cancer (Cha et al., 2008; Riss et al., 2007). Plant based supplements including the algal-based chlorella, spirulina and kelp (*Ascophyllum nodosum*) are being marketed to consumers as a natural source of vitamins, minerals, carotenoids, proteins and other bioactive compounds (Gouveia et al., 2008), with associated benefits to health.

Chlorella are a class of unicellular green microalgae. They are species that grow in fresh and marine water (Wang et al., 2010). Supplementation with chlorella has been found to have anti-cancer effects in rats (Amin, 2009), cognitive protective effects in mice (Nakashima et al., 2009) and gamma radiation protective effects in mice (Singh *et al*., 1995). Extracts from chlorella have also exhibited anti-cancer effects in a range of cell models (Cha et al., 2008; Wang et al., 2010). Spirulina is a blue-green cyanobacterium species of which grows in both fresh or seawater and have been found to contain a wide range of nutrients and bioactives as well as very high levels of protein (Karkos et al., 2011). Supplementation with fresh or dried spirulina has been found to exhibit immunomodulatory effects (Mao et al., 2005) and protective effects against arsenicosis (Rahman et al., 2008) in humans, and have demonstrated atherosclerosis preventive effects in rats (Riss et al., 2007). Extracts from spirulina have exhibited anti-cancer (Li et al., 2010) and anti-viral effects (Chirasuwan et al., 2009) in *in-vitro* models. *A. nodosum* is a brown seaweed distributed throughout the Atlantic coasts of Europe and America. Extracts from *A. nodosum* have shown anti-cancer and anti-diabetic effects in *in-vitro* models (Nwosu et al., 2011).

The objective of this study was to compare the antioxidant activity of methanolic extracts from commercially sourced powders *Chlorella vulgaris* (fresh water), *A. nodosum* (marine) and *Spirulina platensis* (fresh water). The antioxidant potential of the extracts was assessed by measuring the total phenol content (TPC), the ferric reducing antioxidant potential (FRAP); and the ability to inhibit β-carotene bleaching (β-CB). The antioxidant status in human adenocarcinoma cells, Caco-2 was assessed following incubation with the supplement extracts by measuring the activity of antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) and

the reduced glutathione (GSH) content. The ability of the extracts to protect against hydrogen peroxide (H_2O_2) and *tert*-Butyl hydroperoxide (*tert*-BOOH)-induced depletion of SOD was assessed and the potential protective effects of the extracts against H_2O_2 and *tert*-BOOH induced DNA damage was determined in Caco-2 cells using the comet assay. Caco-2 cells are a colon cancer cell line commonly used as a human cell model to predict the possible biological effects of food supplements and drugs on intestinal cells.

2. Materials and Methods

2.1. Materials

Human colon adenocarcinoma, Caco-2 cells, were obtained from European Collection of Animal Cell cultures (Salisbury, UK). Foetal bovine serum (FBS) was obtained from Invitrogen (Paisley, Scotland). Cell culture plastics were obtained from Cruinn Diagnostics (Greiner Bio-One, Frickenhausen, Germany). SOD Assay Kit II (Calbiochem) and Catalase Colorimetric Activity Kit (Calbiochem) were purchased from Merck Chemicals Ltd. (Nottingham, UK). All other cell culture reagents and chemicals were purchased from Sigma Chemical Co. (Dublin, Ireland).

2.2. Preparation of algal extracts

A. nodosum (Kelp, Irish Health Foods Ltd., Cork, Ireland); *C. vulgaris* (Chlorella, Life Stream, Auckland, New Zealand) and *S. platensis* (Spirulina, Synergy Natural, Australia) powders were purchased from a local health food store (Natural Choice, Cork) in October 2010. The powders each consisted of 100% pure dried algae. The recommended daily intake was between 1-3 g, to be consumed in fruit juice or smoothies. An extract of each of the seaweed supplements was prepared using the method of Connan et al. (2004) with slight modifications. Briefly, samples (10 g) were ground with a pestle and mortar, and then mixed with 30 ml of 60% aqueous methanol. The mixture was homogenised at 24,000 rpm for 1 min, placed in the dark at 37˚C for 3 hrs and centrifuged at 9,000 rpm for 15 mins then filtered with Whatman no. 1 filter paper. Methanol was removed from the resulting supernatant via solvent evaporation. Extracts were freeze-dried and then weighed. The extraction yield (%) was calculated as follows:

Extraction yield, % = (Weight of extract / Weight of original sample) × 100

The dried samples were reconstituted in distilled water to a final concentration of 10 mg/ml.

2.3. Antioxidant potential of the seaweed supplement extracts

TPC, FRAP and β-CBA were carried out as described in O'Sullivan et al. (2011). TPC was determined using the Folin-Ciocalteau method. Folin-Ciocalteau's phenol reagent (250 µl) and 500 µl Na_2CO_3 (2% w/v) were added to 50 µl of each sample. After 5 min, 4.2 ml H_2O was added. Samples were incubated at 25°C for 2 hrs. The absorbance was determined at 765 nm (WPA Lightwave S2000, Cambridge, UK).

Gallic acid was used to produce a calibration curve and the total phenol content of the sample extracts were expressed as mg gallic acid equivalents/g dry weight of extract (GAE/gdw). The FRAP assay determines the antioxidant potential of the samples by measuring the reduction in ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). FRAP reagent [2 ml; 0.01M TPTZ (2,4,6-tripyridyl-s-triazine) in 0.04M HCl, 0.02M $FeCl_3.6H_2O$ and 0.3M acetate buffer] was added to 800 µl distilled H_2O and 200 µl of each sample. A standard curve was constructed using $FeSO_4.7H_2O$ (Fe^{2+}). Ascorbic acid was used for comparison. Following 30 mins the absorbance was measured at 593nm (WPA Lightwave S2000, Cambridge, UK). The results were expressed as µM ascorbic acid equivalence/mg sample (µM AAEq/mg). The ability of the extracts to prevent β-carotene bleaching (β-CB) was measured according to the method of Duan et al. (2006). Briefly, a mixture of oxygenated 100 ml H_2O, 4 mg β-carotene, 400 mg Tween-40 and 40 mg linoleic acid was incubated with or without 50 µl of sample for 180 min at 50°C and the absorbance was measured at 450 nm at 30 mins intervals (WPA Lightwave S2000, Cambridge, UK). The results were expressed as % β-CB inhibition.

2.4. Cell culture

Human colon adenocarcinoma Caco-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) enriched with FBS (10% v/v) and non-essential amino acids (1% v/v). Cells were incubated in an atmosphere of CO_2–air (5:95, v/v) at 37°C and were maintained in the absence of antibiotics. To determine the appropriate non-toxic concentrations of the extracts for addition to cells, Caco-2 (1 x 10^5 cells/ml) were first exposed to 0.05-0.5 mg/ml of the extracts for 24 hrs in 96-well plates with a final volume of 100 µl. Cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay (MTT I proliferation kit, Roche Diagnostics; Burgess Hill, West Sussex, UK).

2.5. Cellular antioxidant status

Caco-2 cells (1 x 10^5 cells/ml) were incubated with 0.05 mg/ml *C. vulgaris* or *A. nodosum* or 0.15 mg/ml *S. platensis* for 24 hrs in a 6 well plate and the antioxidant status of the cells was determined by measuring GSH content, catalase activity and SOD activity. GSH was determined by the method of Hissin & Hilf (1976). Briefly, cells were sonicated and then centrifuged at 14,000 rpm for 30 mins at 4°C. 100 µl

of the resulting supernatant was added to a mixture of sodium phosphate-ethylenediamine tetraacetic acid buffer and o-phthaldialdehyde (1 mg/ml). Fluorescence was detected at 430 nm following excitation at 360 nm (Tecan SpectraFluor Plus, Unitech Ltd, Ireland). GSH levels (nmol) were expressed relative to the protein content (mg), as determined by the bicinchoninic acid (BCA) method (Smith et al., 1985). Catalase and SOD activity were measured according to the instructions supplied with their respective kits. The SOD activity was also assessed in Caco-2 cells pre-incubated with the extracts for 24 hrs prior to exposing the cells to either 200 μM H_2O_2 or 1 mM *tert*-BOOH for 30 mins at 37°C.

2.6. Determination of DNA damage (Comet assay)

Caco-2 cells (1 x 10^5 cells/ml) were supplemented with 0.05 mg/ml *C. vulgaris* or *A. nodosum* or 0.15 mg/ml *S. platensis* for 24 hrs in 6-well plates to a final volume of 2 ml. Following incubation, the cells were treated with or without 50 μM H_2O_2 or 200 μM *tert*-BOOH for 30 mins at 37°C. Cells were harvested, embedded in a low melting point agarose (1%) and were placed on microscope slides coated with a layer of normal gelling agarose. Slides were placed in lysis solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 1% (w/v) sodium sarcosinate, 1% (v/v) Triton X-100 and 10% (v/v) DMSO; 4°C] for 2 hrs. Slides were then placed in a horizontal gel electrophoresis tank (Horizon 20.25, GIBCO BRL, Life Technologies, Paisley, Scotland) containing fresh electrophoresis solution (300 mM NaOH, 1 mM EDTA) for 30 mins. Electrophoresis was performed for 25 mins at 4°C using a 25 V (300 mA) current. Following electrophoresis, the slides were washed with neutralising buffer (0.4 M Tris, pH 7.5). Slides were stained with ethidium bromide (20 μg/ml), then covered with coverslips. Cells were visualised at a magnification of 200 x (Nikon Optiphot) and the Komet 5.5 image analysis software program (Andor Technology, Belfast, Northern Ireland) was used to determine the level of DNA damage which was expressed as percentage tail DNA.

2.7. Statistical analysis

Data represent the mean ± se of 4 independent experiments. Statistical analysis was by repeated measures ANOVA followed by Dunnett's or Tukey's test (Prism 4.0, GraphPad Inc, San Diego, CA, USA). The level of statistical significance was taken at $P < 0.05$ or $P < 0.01$.

3. Results and Discussion

3.1. Extraction yield

Each of the seaweed powders was extracted using a 60% methanol aqueous solution to produce an antioxidant enriched fraction. The yields of *C. vulgaris*, *S. platensis* and *A. nodosum* were 2%, 8.5% and 7%, respectively, following extraction. The quantity and profile of antioxidants extracted is dependent on the solvent polarity. The greatest antioxidant yields are generally achieved using either ethanol or methanol or their aqueous mixtures (Franco et al., 2008). The phenolic content of the *C. vulgaris*, *A. nodosum* and *S. platensis* extracts were 14, 154 and 12 mg GAE/gdw (Table 1). The yields from a commercial spirulina powder were reported to be 25.5% when extracted using water and 12.8% using a dichloromethane: methanol (1:1) mixture, the total phenol content of the extract was 16 mg GAE/gdw, similar to that of the present study (Shukla et al., 2009). A pressurized liquid extraction (PLE) which involves the use of conventional solvents at elevated temperatures and pressures has previously been used to extract an antioxidant enriched fraction from *C. vulgaris*, the yield ranged from 15% to 40%, increasing as the temperature was elevated, up to 160°C, the total phenol content of the extracts peaked at 15 mg GAE/gdw (Cha et al., 2010). Brown algae such as *A. nodosum* contain higher polyphenol levels than green algae. The polyphenol content of *A. nodosum* has been shown to vary between 2% and 14% of the dry matter dependent on environmental factors such as season and salinity of the habitat (Ragan & Jensen, 1978), therefore the results of our study are just slightly higher than the expected range. In a previous study we found that the TPC of a methanolic extract of *A. nodosum*, harvested from the Irish coastline, was 4.5 mg GAE/g dry seaweed weight (O'Sullivan et al., 2011).

3.2. *In-vitro* antioxidant assays

The FRAP activities of *C. vulgaris*, *A. nodosum* and *S. platensis* were found to be 339, 616 and 297 μM AAEq, respectively (Table 1). *A. nodosum* had a twofold higher FRAP activity compared with *C. vulgaris* and *S. platensis* which correlated with its higher TPC. A hexane/ ethyl acetate/ water extract of *Chlorella vulgaris* has previously shown a FRAP value of approximately 58 μmol Trolox/ g sample (Hajimahmoodi et al., 2009). Extracts from *C. vulgaris*, *A. nodosum* and *S. platensis* were found to reduce β-CB by 100%, 64% and 94%, respectively (Table 1).

Mendiola et al. (2007) measured the antioxidant activity of *S. platensis* extracted by supercritical fluid at various parameters including extraction pressure, temperature and solvent concentration, they found a wide variability in the β-CB activity (1%-97%) depending on the parameters selected for the extraction procedure. Our previous study found that a methanol extract from *A. nodosum* reduced β-CB by 76% (O'Sullivan et al., 2011). Results obtained for the β-CB inhibition assay did not correlate with those of the TPC or FRAP activity assays which may be as a result of the fact that β-CB is only inhibited by non-polar antioxidant compounds such as carotenoids and not polar compounds such as polyphenols (Chew et al., 2008).

Table 1

Total Phenol Content (TPC), Ferric Reducing Antioxidant Potential (FRAP) assay, and β-Carotene Bleaching (β-CB) Assay of algae extracts.

	C. vulgaris[(a)]		*A. nodosum*[(b)]		*S. platensis*[(c)]	
	Mean	se	Mean	se	Mean	se
TPC (mg gallic acid equivalent)	14.0[b]	0.5	154.0[a,c]	1.5	12.0[b]	0.5
FRAP (μM ascorbic acid equivavent)	339.0[b]	9.5	616.0[a,c]	41.0	297.0[b]	31.0
BCBA (% protection)	100.0[b]	9.5	64.0[a,c]	4.5	94.0[b]	5.5

[(a-c)] Denote a significant difference between mean values, where [a] denotes a significant difference from *Chlorella vulgaris*, [b] denotes a significant difference from *Ascophyllum nodosum* and [c] denotes a significant difference from *Spirulina platensis*. Repeated measures ANOVA followed by Tukey's post-test. n= 4 independent experiments.

3.3. Antioxidant status in Caco-2 cells

The effects of the supplement extracts on cell viability were assessed at concentrations between 0.05 and 5 mg/ml using the MTT assay (Figure 1). Sub-toxic concentrations were determined for each extract (*C. vulgaris*, 0.05 mg/ml; *A. nodosum*, 0.05 mg/ml; *S. platensis*, 0.15 mg/ml) and these concentrations were used for the remainder of the study. At the selected concentrations the seaweed supplement extracts did not affect SOD or CAT activity (Figure 2) nor did the extracts protect against the 20% decrease in SOD which was induced by either H_2O_2 or *tert*-BOOH in Caco-2 cells (Table 2). A six week chlorella supplementation (6.3g per day) was shown to increase the CAT and SOD activity in the erythrocytes of male Korean smokers (Lee et al., 2010) and spirulina increased the activity of CAT and SOD in the liver of Swiss albino mice at both 250 and 500 mg/kg body weight (Dasgupta et al., 2001). It is possible that chlorella and spirulina may be metabolised *in-vivo* resulting in the formation of metabolites which enhance the activity of cellular antioxidant enzymes as there was no effect observed on the antioxidant enzyme activity in Caco-2 cells in the present study (Figure 2). Alternatively, the concentrations of the supplement extracts added to the cells may not have been within the effective range. Spirulina supplementation increased the content of GSH in the liver of Swiss albino mice (Dasgupta et al., 2001), we also observed a significant ($P < 0.05$) increase in the GSH content of Caco-2 cells following incubation with extracts prepared from *C. vulgaris* and *S. platensis* (Figure 2). An increase in the content of glutathione could indicate an increase in the activity of the enzyme glutathione reductase which converts oxidized GSH to reduced GSH. The GSH content was decreased significantly ($P < 0.01$) following incubation with *A. nodosum* (Figure 2). A reduction in the cellular content of glutathione was also observed in human blood cells collected from volunteers who had consumed powdered *Spirulina maxima* for a 30 day period (Shyam et al., 2007). Studies have shown that compounds such as L-buthionine-S,R-sulfoximine (BSO) which reduce GSH in cancer cells can sensitize the cells to chemotherapeutic agents, however further investigation would be required before a role for *A. nodosum* (which also depleted GSH) in the treatment of cancer treatment could be proposed.

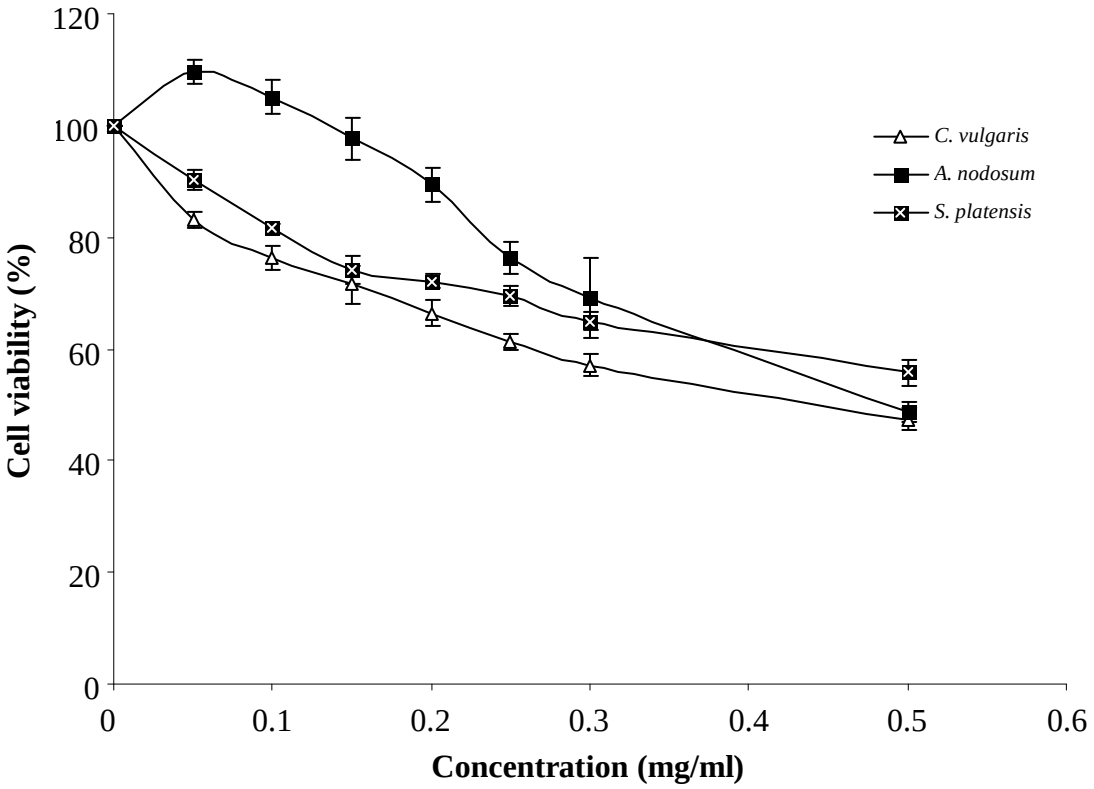


Figure 1: Viability of Caco-2 cells following addition of increasing levels (mg/ml) of *Chlorella vulgaris* (-Δ-), *Ascophyllum nodosum* (-■-) and *Spirulina platensis* (-×-) at concentrations ranging from 0.05-0.5 mg/ml. Data are the means of six independent experiments, with standard errors represented by vertical bars.

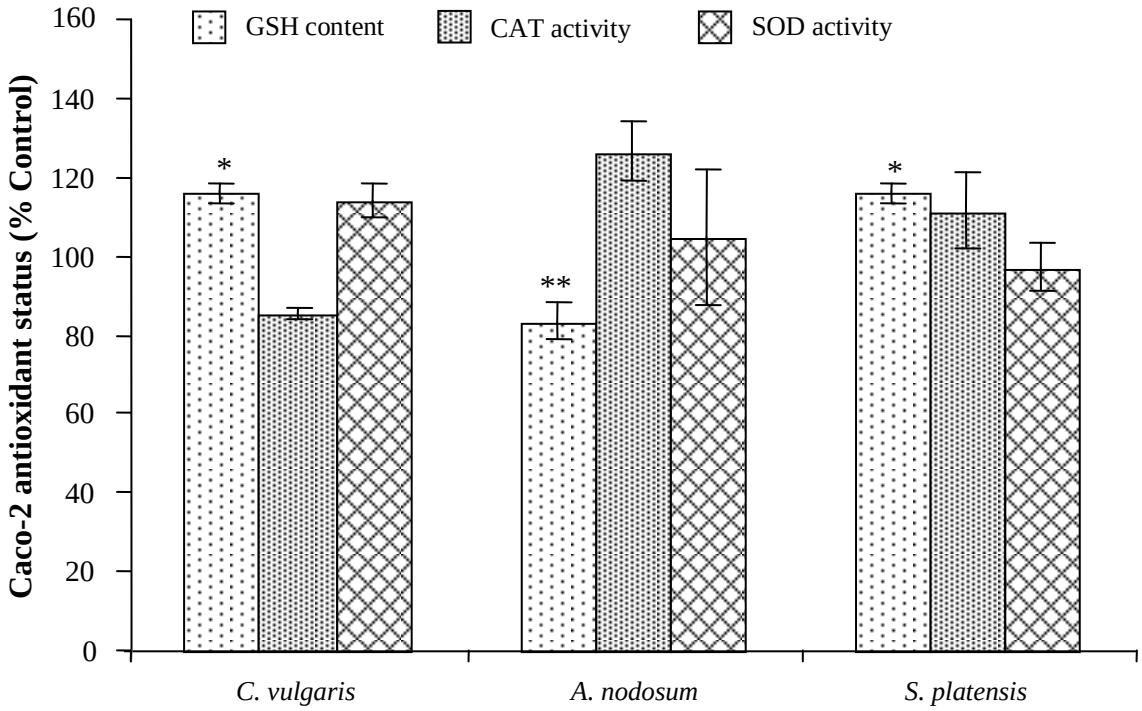


Figure 2: GSH content, catalase and superoxide dismutase activity in Caco-2 cells supplemented with methanolic extracts of *Chlorella vulgaris, Ascophyllum nodosum* and *Spirulina platensis* for 24 hrs. Data are the means of three independent experiments, with standard errors represented by vertical bars. ** Mean value was significantly different from that of the control ($P < 0.01$), * Mean value was significantly different from that of the control ($P < 0.05$), ANOVA followed by Dunnett's test.

Table 2

The potential protective effects of methanolic extracts from *Chlorella vulgaris*, *Ascophyllum nodosum* and *Spirulina platensis* against H_2O_2 and *tert*-BOOH induced modifications of SOD activity in Caco-2 cells.

	SOD activity (%)	
Samples	H_2O_2	*tert*-BOOH
Control	100.0	100.0
Oxidant control	79.0 ± 4.5	80.5 ± 2.5
C. vulgaris	77.0 ± 8.5	84.5 ± 4.5
A. nodosum	82.0 ± 4.0	86.5 ± 6.5
S. platensis	79.5 ± 6.5	82.5 ± 2.5

Repeated measures ANOVA followed by Tukey's post-test. n= 4 independent experiments.

3.4. Protection against oxidant induced DNA damage

The addition of 50 μM H_2O_2 increased DNA damage in Caco-2 cells from a background level of 10% to 55% (Figure 2). None of the extracts significantly protected against H_2O_2-induced DNA damage. The concentration of the extracts which could be added to the cells was limited by the cytotoxic effects exhibited at higher concentrations and the polyphenol concentration added was approximately 0.7 μg/ml, 7.7 μg/ml or 1.8 μg/ml for *C. vulgaris*, *A. nodosum* and *S. platensis*, respectively. The polyphenol epicatechin gallate has demonstrated a protective effect against H_2O_2 induced DNA damage in C6 astroglial cells at concentrations of 0.05 and 0.5 μg/ml but was pro-oxidant at higher concentrations (Abib et al., 2010). 2,7''-phloroglucinol-6,6'-bieckol (PHB) a phlorotannin isolated from the brown algae, *Ecklonia cava*, protected against H_2O_2 induced DNA damage in Vero cells at concentrations exceeding 25 μg/ml (Kang et al., 2012). A hot water extract of *C. vulgaris* has demonstrated a protective effect against H_2O_2-induced DNA damage in human skin fibroblasts (Makpol et al., 2009) and a purified peptide isolated from *C. vulgaris* protected against hydroxyl radical induced DNA damage in human lung fibroblast cells (Sheih et al., 2009). The addition of 200 μM *tert*-BOOH to Caco-2 cells increased DNA damage to 30%. The *S. platensis* extract significantly protected against *tert*-BOOH induced DNA damage and reduced DNA damage to 20%. Neither the *A. nodosum* nor *C. vulgaris* extract protected against *tert*-BOOH induced DNA damage.

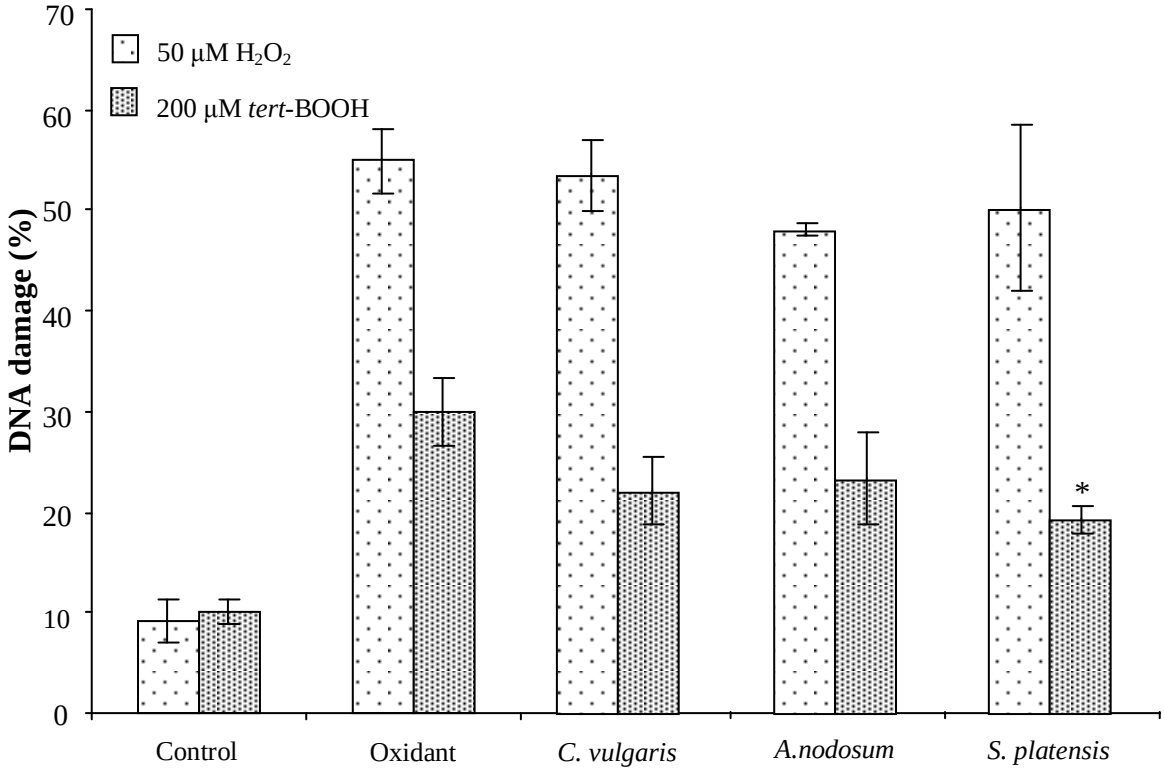


Figure 2: DNA damage in Caco-2 cells, expressed as percent tail DNA. Cells were incubated with methanolic extracts of *Chlorella vulgaris*, *Ascophyllum nodosum* and *Spirulina platensis* for 24 hrs, media was removed and replaced with media containing 50 μM H_2O_2 or 200 μM *tert*-BOOH for a further 30 mins. Data are the means of four independent experiments, with standard errors represented by vertical bars. * Mean value was significantly different from that for H_2O_2 or *tert*-BOOH, ANOVA followed by Dunnett's test.

4. Conclusions

Each of the supplements exhibited a potent antioxidant potential, especially the *A. nodosum* extract in terms of polyphenol content and FRAP activity, while the *C. vulgaris* and *S. platensis* extracts exhibited the best β-CB inhibition activity. The results, especially the β-CB, may be of interest to the food industry in terms of preventing or reducing lipid rancidity in foods. In the cellular model *C. vulgaris* and *S. platensis* significantly increased GSH content while *A. nodosum* significantly decreased GSH content. In addition the *S. platensis* extract significantly reduced *tert*-BOOH -induced DNA damage. However none of the extracts affected antioxidant enzymes or protected against H_2O_2-induced DNA damage. The minimal antioxidant activity exhibited by the extracts in the current study may suggest that the supplements are more effective their whole form as opposed to isolated extract form. Further analysis is required to determine the possible health benefits of these supplements *in-vivo* and the subsequent potential pharmaceutical derived compounds from these seaweed derived nutritional supplements.

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CHAPTER 8

General discussion

Seaweed is a rich source of antioxidant compounds such as polyphenols, sulphated polysaccharides, carotenoids and many other compounds. Seaweed-derived polyphenols are considered to be superior to the polyphenols found in terrestrial plants due to their complex ring structure (Burtin, 2003). The objective of this thesis was to investigate the antioxidant activity of extracts prepared from a range of brown seaweeds, harvested from the Irish coast, and commercially available algae based supplements using both *in-vitro* and cell model-based models, with the overall view of producing potential functional ingredients for incorporation into dairy products such as yogurt and milk.

Initially, the antioxidant activity of 60% methanol (MeOH) extracts from *Ascophyllum nodosum, Laminaria hyperborea, Pelvetia canaliculata, Fucus vesiculosus* and *Fucus serratus* were compared and it was found that all extracts exhibited strong antioxidant activity. In general, the *F. vesiculosus* and *F. serratus* extracts exhibited the greatest antioxidant activity. The *F. vesiculosus* extract was most effective at preventing β-carotene bleaching and scavenging DPPH radicals while the *F. serratus* extract contained the highest total phenol content. Both extracts exhibited similar ferric reducing antioxidant power (FRAP) activity (Chapter 2). A cell model system, the human colon adenocarcinoma (Caco-2) cell line was used to further investigate the antioxidant potential of the seaweeds. All five seaweeds enhanced the cellular content of the antioxidant glutathione (GSH) and protected against depletion of the antioxidant enzyme, superoxide dismutase (SOD) but only *F. vesiculosus* and *F. serratus* extracts protected against the DNA damage induced by the oxidant hydrogen peroxide (H_2O_2). The ability of the seaweeds to protect against the oxidative stress induced by a second oxidant, tert butyl hydroperoxide (*tert*-BOOH) in Caco-2 cells was then investigated (Chapter 3). *L. hyperborea, P. canaliculata and F. serratus* extracts protected against a *tert*-BOOH induced decrease in SOD activity but none of the five extracts protected against the *tert*-BOOH induced increase in GSH or the reduction in CAT activity. *F. vesiculosus* and *F. serratus* were selected for further investigation and their ability to protect against DNA damage induced by H_2O_2 and *tert*-BOOH was compared with that of three metal chelators deferoxamine mesylate (DFO), 1, 10-phenanthroline (o-phen) and 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (BAPTA-AM). *F. vesiculosus* and *F. serratus* protected against H_2O_2 but not *tert*-BOOH induced DNA damage. Each of the metal chelators protected against *tert*-

BOOH induced DNA damage which indicates that the metal chelating capacity of *F. vesiculosus* and *F. serratus* was not sufficient to protect against the DNA damage induced by this oxidant.

To determine an optimal method for the extraction of antioxidants from seaweed, three of the seaweeds *A. nodosum*, *F. serratus* and *F. vesiculosus* were selected and an extract was prepared using 100% water; 60% ethanol (EtOH) (v/v); 80% EtOH (v/v) and 60% MeOH (v/v), in combination with an accelerated solvent extraction (ASE®) technique (Chapter 4). None of the resulting extracts altered the antioxidant status (SOD activity, CAT activity, GSH content) of oxidant (H_2O_2) challenged or unchallenged Caco-2 cells. These findings suggest that the accelerated solvent extraction method employed may have damaged certain bioactives responsible the antioxidant activity displayed Chapter 2 and 3. High temperature extraction processes have been reported to influence the content and activity of potential antioxidant compounds sourced from plant material due to the thermal damage of various compounds (Larrauri et al., 1997). A number of the ASE®-derived extracts did however protect against H_2O_2 and *tert*-BOOH-induced DNA damage in Caco-2 cells indicating that the extracts did possess some antioxidant activity. Extracts prepared from *A. nodosum* using 100% water (AN_{100}) or 80% ethanol (AN_{80e}) and *F. vesiculosus* using 60% ethanol (FV_{60e}) were the extracts which demonstrated the greatest antioxidant effect against oxidant-induced DNA damage and these extracts were selected for addition to dairy products in a further study.

Chapters 5 and 6 required the use of a large volume of seaweed extract as an ingredient in milk and yogurt which made the ASE® system, which produces extracts on a small-scale, unviable and instead a solid liquid extraction method was employed and the resulting extracts were added to milk at concentrations of 0.25% and 0.5%. Seaweed addition did not affect the pH or microbial quality but did influence the colour of the resulting milk by a sensory panel. Milk samples containing AN_{80e}, and FV_{60e} at both the 0.25% and the 0.5% concentrations had higher greenish values than the other samples while milk containing AN_{80e} (0.25%), AN_{80e} (0.5%), FV_{60e} (0.25%), and FV_{60e} (0.5%) had higher yellowness than other samples. The perception of a fishy taste was the determining factor in the negative perception of milk. A fishy taste was detected in milk containing AN_{80e} (0.5%) and FV_{60e} (0.25%) and these samples were found to be the least overall accepted samples by panellists. The unsupplemented control and the milk containing AN_{100} (0.5%) were the most

overall accepted milk samples. Seaweed extract supplemented milk was found to have significantly higher DPPH scavenging activity compared to the control. Despite a decrease in the DPPH activity of the AN_{80e} (0.5%) sample following digestion the DPPH activity of most milk samples was stable during digestion. Milk or milk digestates did not protect against H_2O_2-induced DNA damage or influence the antioxidant status in Caco-2 cells.

A similar investigation was conducted by adding the seaweed extracts to yogurt. The addition of seaweed did not significantly affect the bacterial content, pH, whey separation or rheological properties of the yogurts. Seaweed enrichment did however influence the colour of the resulting yogurts. Yellowness values were significantly higher in yogurts containing the FV_{60e} extracts at both concentrations and AN_{80e} at the 0.5% concentration. Lipid oxidation was significantly lower in yogurts containing 0.5% AN_{80e} and 0.5% FV_{60e}. Sensory analysis revealed that appearance and flavour governed the overall acceptability of yogurts with the control yogurt and yogurts containing 0.25% AN_{100} and 0.5% AN_{100} being the most preferred samples by sensory panellists. Seaweed-enriched yogurts had significantly higher DPPH scavenging activity than unsupplemented samples and this activity remained stable overtime. To assess bioactivity, the yogurt samples were subjected to an *in-vitro* digestion procedure, which mimics human gastric and duodenal digestion and the antioxidant activity of the yogurt and yogurt digestates was assessed. It was found that, with the exception of the sample enriched with 0.5% FV_{60e}, the DPPH scavenging activity of the yogurts was decreased following digestion. Yogurt and digested yogurt samples did not affect the antioxidant status (CAT activity, SOD activity and GSH content) or protect against H_2O_2-induced DNA damage in Caco-2 cells. Therefore, our findings suggest that the addition of seaweed extracts improves the shelf-life and quality attributes of yogurts but the supplemented yogurts do not protect against oxidant induced DNA damage in Caco-2 cells under the conditions of our experiment.

In summary, the present thesis applied *in-vitro* and cell model-based antioxidant assays to screen the antioxidant activity of seaweeds extracted using a variety of solvent systems to establish an optimal extraction technique with the most effective extracts being selected as functional ingredients for addition to milk and yogurt. The addition of seaweed extracts to milk and yogurt improved their shelf-life stability and *in-vitro* antioxidant activity but did not enhance antioxidant activity

in Caco-2 cells, under the conditions of our experiments. The use of animal and human trials may be required to fully investigate the health benefits of seaweed-enriched dairy products. In addition the negative impact of some seaweed extracts, especially in yogurt, on the colour and flavour of the resulting products must to be addressed through the use of flavourings and colourings, or more advanced methods such as microencapsulation or similar techniques, before these products can potentially be produced on a commercial scale.

A recent market research study compiled by Global Industry Analysts, Inc. (November, 2012) estimates that the global functional foods and beverages market is set to exceed $130 billion by 2015. These market projections are based on growth drivers such as increasing health awareness, rising disposable incomes and an aging world population. A study by Jaspars and Folmer (2013) illustrates the large number of seaweed enriched food products marketed globally including products such as seaweed enriched table salt, probiotic drinks, potato chips and other products indicating the potential of Irish seaweed as a functional food ingredient. According to the Global Industry Analysts, Inc. (November, 2012) market study the functional food market is currently dominated by food products enriched with ingredients with probiotic, prebiotic, and cholesterol lowering functional properties. A number of seaweed-derived ingredients have also exhibited prebiotic and cholesterol-reducing properties, as well as antioxidant and anti-diabetic properties, therefore seaweed extracts could be potentially used to enrich a wide variety of everyday food products. According to Guiry (www.seaweed.ie) the main obstacle inhibiting the extensive use of seaweed-derived ingredients in functional foods is that many consumers perceive seaweed negatively. To counter this Guiry (www.seaweed.ie) suggests that seaweed and seaweed-derived ingredients should be marketed as sea vegetables. The results of the present PhD (funded as part of the NutraMara project which investigated the potential use of marine-derived products as functional ingredients) illustrate that seaweed-derived antioxidants can be successfully used to produce antioxidant fortified milk and yogurt products without overly affecting the quality, shelf-life and sensory properties. Continued scientific and market research is required to fully determine the marketability of seaweed enriched functional products (Figure 1) and to fully substantiate its health claims. A potential schematic for the production of seaweed-enriched dairy products is outlined in Figure 2.



Figure 1. A possible future concept product enhanced with seaweed extracts.

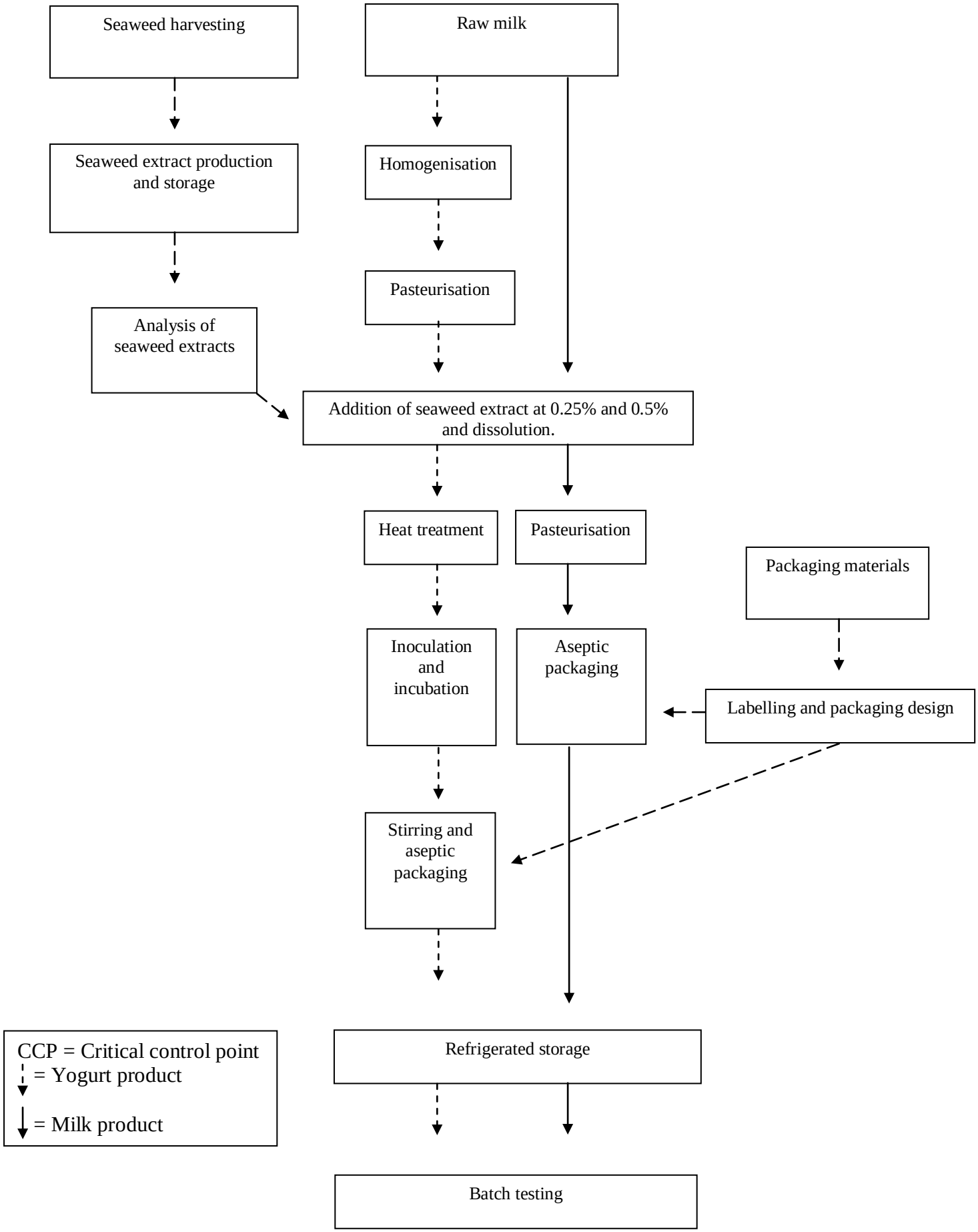


Figure 2. A proposed schematic for the large scale production of seaweed-enriched dairy products.

References

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