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MACROALGAE AND COMMERCIAL MACROALGAL POLYSACCHARIDES AS POTENTIAL FUNCTIONAL INGREDIENTS IN MUSCLE FOODS

A Thesis submitted in the fulfilment of the requirements for the degree of Doctor of Philosophy (PhD, Food Science and Technology)

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Dedicated to my Gpa & Gma

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Declaration

I hereby declare that this thesis is my own work and contains no material that has been accepted for the award of any other degree or diploma in any university.

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgement has been made.

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Abstract

Ulva rigida (UR) and *Palmaria palmata* (PP) were included in farmed Atlantic salmon diets at levels of 0-15% for 19 and 16 weeks, respectively. Quality and shelf-life parameters of salmon fillets stored in modified atmosphere packs (MAP) (60% N_2 : 40% CO_2) at 4ºC were compared to controls fed astaxanthin. Salmon fillets were enhanced with a yellow/orange colour. Proximate composition, pH and lipid oxidation were unaffected by dietary UR and PP. Salmon fed 5% UR and 5-15% PP did not influence sensory descriptors (texture, odour, oxidation flavour and overall acceptability) of cooked salmon fillets.

Pig diets were supplemented with commercial wet- and spray-dried macroalgal (*Laminaria digitata*) polysaccharide extracts containing laminarin (L, 500 mg/kg feed) and fucoidan (F, 420 mg/kg feed) (L/F-WS, L/F-SD) for 3 weeks and quality and shelf-life parameters of fresh pork steaks (*longissimus thoracis et lumborum*) stored in MAP (80% O_2 : 20% CO_2) were examined. Level (450 or 900 mg L and F/kg feed) and duration (3 or 6 weeks) of dietary L/F-WS and mechanisms of antioxidant activities in pork were investigated. L/F-WS reduced ($p < 0.05$) lipid oxidation and lowered levels of saturated fatty acids in fresh pork after 3 weeks feeding.

L/F-SD was added directly to mince pork (0.01 - 0.5%) and quality and shelf-life parameters of fresh pork patties stored in MAP (80% O_2 : 20% CO_2) were assessed. Direct addition of the L/F-SD increased levels of lipid oxidation and decreased surface redness (a* values) of fresh pork patties. Lipid oxidation was reduced in cooked patties due to the formation of Maillard reaction products. Cooked pork patties containing L/F-SD were subjected to an *in vitro* digestion and a cellular transwell model to confirm bioaccessibility and uptake of antioxidant compounds. In mechanistic studies, fucoidan demonstrated anti- and pro-oxidant activities on muscle lipids and oxymyoglobin, respectively.

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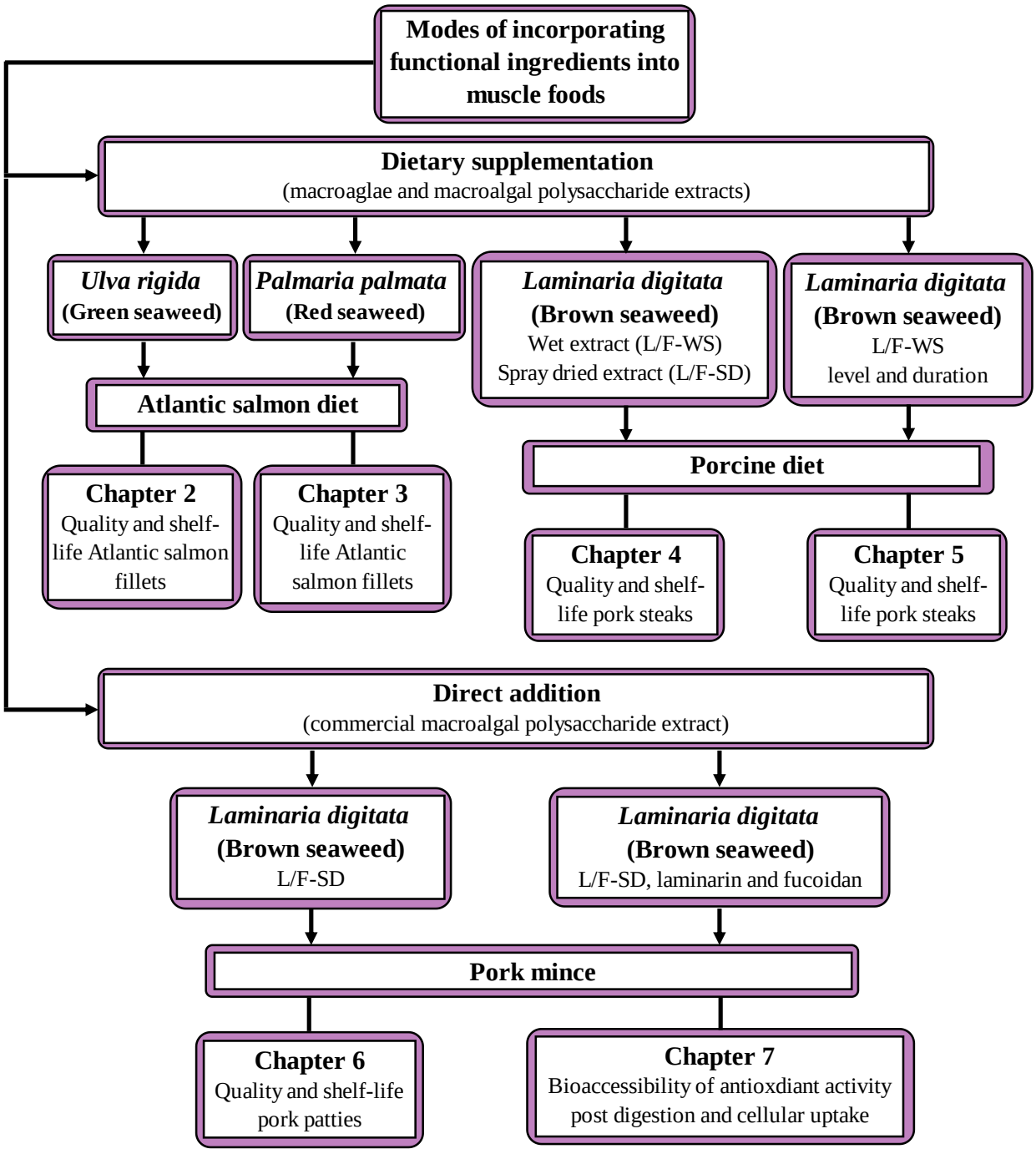
Thesis Overview Schematic



List of Abbreviations

4-HNE - 4-hydroxy-2-nonenal
8-ohdG - 8-hydroxydeoxyguanosine
ASE® - accelerated solvent extraction
ABTS - 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
BCBA - β-carotene bleaching assay
BHA - butylated hydroxyanisole
BHT - butylated hydroxytoluene
CACO-2 - cultures of human colorectal cancer epithelial cell lines
CAT - catalase
DHA - docosahexaenoic acid
DM - dry matter
DPPH - 2,2-diphenyl-1-picrylhydrazyl
EAE - enzyme assisted extraction
EC_{50} - half maximal effective concentration
EIA - enzyme immunoassay
ELISA - enzyme-linked immunosorbent assay
EPA - eicosapentanoic acid
ESR - electron spin resonance spectroscopy
ET - electron transfer
FAMEs - fatty acid methyl esters
FICA - ferrous ion chelating activity
FRAP - ferric reducing antioxidant power
GC - gas chromatography
GC-MS - gas chromatography-mass spectrometry
GIT - gastrointestinal tract
GSH - glutathione
GSHPx - glutathione peroxidise
HAT - hydrogen atom transfer
HPLC - high-performance liquid chromatography
LDL - low density lipoprotein
L/F - laminarin/fucoidan
L/F_{450-3} - laminarin/fucoidan level (450 mg/kg feed) and duration (3 weeks)
L/F_{900-3} - laminarin/fucoidan level (900 mg/kg feed) and duration (3 weeks)
L/F_{450-6} - laminarin/fucoidan level (450 mg/kg feed) and duration (6 weeks)
L/F_{450-6} - laminarin/fucoidan level (900 mg/kg feed) and duration (6 weeks)
L/F - SD - spray-dried seaweed extract containing laminarin and fucoidan
L/F-WS - wet-extract containing laminarin and fucoidan
LLE - liquid–liquid extraction
LTL - *longissimus thoracis et lumborum*
MAE - microwave-assisted extraction
MAP - modified atmosphere packs
MDA - malondialdehyde
MRPs - Maillard reaction products
ORAC - oxygen radical absorbance capacity
PC - positive control
PCA - standard plate count agar
PG - propyl gallate

PLE - pressurized liquid extraction
PP - *Palmaria palmata*
PUFA - polyunsaturated fatty acid
ROS - reactive oxygen species
SCFA - short chain fatty acid
SFA - saturated fatty acid
SFE - supercritical fluid extraction
SLE - solid–liquid extraction
SOD - superoxide dismutase
Spp. - species
ST - salt control
TAS - total antioxidant capacity
TBA - 2-thiobarbituric acid
TBARS - thiobarbituric acid-reactive substances
TC - tea catechin (positive lipid oxidation control)
TCA - trichloroacetic acid
TEAC - trolox equivalent antioxidant capacity
TOSC - total oxidant scavenging capacity
TPA - texture profile analysis
TPC - total phenol content
TRAP - total radical trapping antioxidant parameter
TVC - total viable count
UAE - ultrasound/sonication extraction
UR - *Ulva rigida*
WEPO® - water extraction and particle formation on-line
WHC - water holding capacity

“Let food be thy medicine, let medicine be thy food” – Hippocrates

CHAPTER 1

Literature Review

SECTION 1: SEAWEED CLASSIFICATION, COMPOSITION AND BIOACTIVITY

1.1 Introduction

The world's ocean covers more than 70% of the earth's surface with an environment containing numerous marine photosynthetic organisms. Marine macroalgae, commonly known as seaweeds, are recognized for their richness in polysaccharides, proteins, minerals, vitamins and their low lipid content (1–3% algal dry weight) with high concentrations of certain long-chain polyunsaturated fatty acids (Bocanegra et al., 2009; Fleurence, 1999; Gómez-Ordóñez et al., 2010; Mabeau & Fleurence, 1993). Owing to the harsh environments in which many types of seaweed exist, they produce a range of bioactive compounds as part of an effective defence system. Various biological activities of compounds isolated from marine macroalgae include: antioxidant potential, anti-inflammatory properties, antibacterial, anti-coagulant, anti-viral and apoptotic activities (Gómez-Ordóñez et al., 2014; Mohsen et al., 2007; O'Sullivan et al., 2010; Sinurat & Marraskuranto, 2013; Stengel et al., 2011). Some of the most important biologically active compounds being investigated include: laminarin, fucoidan and polyphenols from brown seaweeds (Zhang et al., 2012).

Among the most relevant compounds sourced from seaweeds, antioxidants are substances that have attracted major interest as of late (Zubia et al., 2009). Antioxidants may have positive health benefits for humans as they protect the body against reactive oxygen species (ROS) which attack membrane lipids, proteins and DNA leading to many health disorders such as a cancer, stroke, diabetes mellitus and is suggested to be the mechanism behind aging (Ngo et al., 2011). Furthermore deterioration of some foods is due to the oxidation of its constituents, e.g. lipids, proteins and minor compounds such as polyphenols (Lund et al., 2011). Oxidation of these compounds can cause a decrease in

nutritional value as well as affecting the safety and appearance of food (Rupérez et al., 2002). The negative effects of oxidation may be mitigated by antioxidants (Chew et al., 2008). Therefore the use of synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG) are extensively used in commercial applications of processing to retard oxidation in foods (Ngo et al., 2011; Pangestuti & Kim, 2011).

In the last few years, the number of publications on natural antioxidant compounds and oxidative stress has nearly quadrupled (Costa et al., 2010). Seaweeds are still considered an underexploited plant resource despite their use for centuries especially by coastal communities (Heo et al., 2005). Motivated by these observations, many researchers have focused in recent years on seaweed compounds, their bioactivities and potential applications as nutraceuticals and functional foods (Shahidi, 2009). A limited number of marine-based functional food products exist covering narrow market niches such as minerals and vitamins from seaweed sources, seaweed protein powders and fibre complexes fortified with photochemical extractions from seaweed (Taylor, 2011).

The addition of functional ingredients with biological activity into processed meats is the subject of much attention, presently (Abu-Ghannam & Cox, 2014). Over the past few decades, meat products have come under scrutiny by consumers due to the association between consumption and the risk of some chronic diseases including heart disease, cancer, hypertension and obesity. Meat based functional foods are seen as an opportunity to address consumer demand for healthier products (Jiménez-Colmenero et al., 2001). The addition of bioactives sourced from seaweeds into meat based foods offers the opportunity to utilise seaweeds and enhance the value of meat for consumers. The incorporation of whole biomass or extracts into muscle based foods, as a high value

functional ingredient, could be used to provide colour, increase nutritional value, improve texture and increase resistance to oxidation (Vo & Kim, 2013).

1.2 Global harvesting, collection and aquaculture production of seaweeds

The marine macroalgae (seaweed) industry has an estimated total annual production value of about US$6 billion. A wide variety of food products contribute about US$5 billion of the total figure (Kraan, 2012). Commercial harvesting of seaweed occurs in about 35 countries globally, in waters ranging from cold to tropical (FAO, 2013). Virtually all brown seaweed, 63% and 68% of red and green seaweeds, respectively are from farmed sources, the majority of which is used for human consumption followed by the extraction of hydrocolloids such as agar and alginate (Marine Foresight Series, 2005). Many types of brown seaweeds such as *Laminaria, Undaria* and *Hizikia* are edible seaweeds mainly used as food, whereas *Sargassum* is primarily used as fertilizer. Popular red seaweeds such as *Phorpyra*, *Gelidium*, *Gracilaria* and *Kappaphycus*/*Eucheuma spp*. are farmed to extract hydrocolloids such as agar and carrageenan (Venugopal, 2008). Seaweeds and seaweed extracts are used in many industries including: animal feed, aquaculture, cosmetics, fuels, wastewater treatment, food supplements and medicinal preparations (Guiry & Guiry, 2014).

The production, marketing and consumption of seaweeds in food products has increased significantly, not only in Asian countries but also in many Western countries, including Ireland, creating a greater demand for seaweed production (Kraan, 2012). In the last fifty years, demand for seaweed has outgrown supply from natural sources (FAO, 2003). Simultaneously, a significant reduction has occurred in seaweed production by traditional Asian producer countries due to environmental pollution, climate change and partial displacement by more profitable finfish and shellfish aquacultures. This trend has

created the market for cultivated (farmed) seaweeds (Tierney et al., 2010). Farm production of seaweeds has expanded since 1970s, with an average global increase of 7.7% annually. To date, more than 14.7 million tonnes of seaweed are commercially produced worldwide, 6% collected from wild stock, 94% from farmed sources. As a result of these factors, efforts have been made in recent years to establish sustainable seaweed aquaculture in Europe and North America (Tierney et al., 2010). An outline of major producers and consumers of seaweeds is presented in Table 1.1.

Table 1.1. Global suppliers and consumers of seaweed.

Country	Supply and consumer facts
Asia-Pacific	Produce 80% world's total supply and the largest consumer base.[a]
China	Largest producer of edible seaweeds (5 million wet tonnes annually).[b] Principle crop: kombu (*Laminaria japonica*).[b]
Republic of Korea	Principle crop: wakame (*Undaria pinnatifida*).[b]
Japan	Largest consumer of edible seaweed (1.6 kg dry wt / person / year).[d] Promotes seaweed as a nutritional foodstuffs.[d] Principle crop: nori (*Porphyra spp.*).[b]
Europe	Farming is not well established.[a]
Ireland, Norway and France	Main producers in the EU.[a]
Spain and Portugal	Small suppliers in the EU.[a]
United Kingdom	Nearly non-existent supply.[a]

[a]FAO (2013).
[b]Marine Foresight series (2005).
[c]Bhattacharjee & Islam (2014).
[d]Cornish & Garbary (2010).

Most seaweed harvested for human consumption grows in marine waters, while the majority of fresh water algae are too toxic as a food source. In general, marine seaweeds are not toxic, however some contain acids that can irritate the digestive tract, or compounds with laxative and electrolyte-balancing effects (Jiménez-Escrig et al., 2012). Seaweeds readily take up and accumulate heavy metals and radioactive materials in areas of pollution and, as a result, may become contaminated and unfit for human consumption

(Werner et al., 2004). A list of edible seaweeds harvested as food stuff and the associated common names are presented in Table 1.2.

1.3 History of seaweed uses and applications

Traditionally, seaweeds have been used world wide as food, fodder and fertilizer and as a source of drugs for medicinal applications since ancient times, particularly in Asian countries and coastal communities (Sánchez-Machado et al., 2004). Seaweed aquaculture in Asia results from specific socio-economic aspects as well as cultural and historical traditions. Natural conditions such as hydro-geography and the abundance of sites available for cultivation have contributed to this development (Werner et al., 2004). In recent years, local governments and commercial organisations in western countries, especially Ireland, France, Canada and the United States, are promoting seaweeds for use in restaurant and domestic purposes, with some success. As people from China, Japan and Korea migrate around the world, the acceptance for seaweed is spreading into the surrounding populations (FAO, 2003). A timeline of seaweed uses throughout history is presented in Table 1.3.

To date, the food sector is still the most important field for application of farmed and wild seaweed species (Buchholz et al., 2012). Presently, 221 species are utilised commercially, including 145 species for food and 110 species for phycocolloid production. The top five cultivated seaweed species in the world are: *Laminaria*, *Porphyra*, *Undaria*, *Eucheuma* and *Garcilari spp*. (Venugopal, 2008). The main uses of green, red and brown seaweed extracts and uses of other seaweed constituents are listed in Table 1.4.

Table 1.2. Scientific and common names of edible seaweed species.

Phylum/Class	Scientific name (genus/species)	Common name
Chlorophyta	*Ulva rigida*[c]	Sea Lettuce
(green)	*Ulva lactuca*[b]	Sea Lettuce
	Ulva spp. (*U. compressa* and *U. intestinalis*)[a]	Sea Lettuce / Sea grass
	Codium spp. (*C. fragile* and *C. tomentosum*)[c]	Spongeweed / Velvet horn
Rhodophyta	*Palmaria palmata*[b]	Dulse
(red)	*Porphyra spp.* (*P. dioica, P. linearis, P. Amplissima* and *P. umbilicalis*)[a]	Nori
	Porphyra spp. (*P. laciniata* and *P. umbilicalis*)[a]	Laver
	Chondrus crispus[b]	Irish moss
	Mastocarpus stellatus[b]	Carrageen moss / False Irish moss / Grape pip weed
Phaeophyceae	(kelps / laminarians)	
(brown)	*Laminaria digitata*[c]	Oarweed / Kelp
	Undaria pinnatifida[b]	Wakame
	Laminaria hyperborea[a]	Tangle / Kelp / May weed
	Saccharina latissima[a]	Sugar kelp
	Saccharina japonica[a]	Kombu
	Alaria esculenta[c]	Badderlocks / Irish Wakame
	(wracks / fucoids)	
	Fucus spiralis[a]	Spiral wrack
	Fucus vesiculosus[a]	Bladderwrack
	Fucus serratus[c]	Toothed wrack / Serrated wrack
	Ascophyllum nodosum[c]	Rockweed / Egg wrack
	Pelvetia canaliculata[c]	Channelled wrack
	(other)	
	Himanthalia elongata[c]	Thongweed / Sea spaghetti
	Sargassum spp. (*S. cinetum, S. vulgare, S. swartzii, S. Muticum* and *S. myriocysum*)[a]	Sargassum / Japanese weed
	Sargassum fusiforme[a]	Hijiki or Hiziki

[a]Guiry & Guiry (2014).
[b]Rhatigan (2009).
[c]Morrissey et al. (2001).

Table 1.3. Uses of seaweeds throughout history.

Time line	Uses of seaweed
Fourth century	Included as part of the local diet in Japan.[a]
Sixth century	Included as part of the local diet in China.[a]
Nineteenth century	Used as soil fertilizer by coastal dwellers.[b]
Thirtieth century	Considered of medicinal value in the Orient.[c]
pre-Christian times	Used as sources of dying agents and anthelmintics in the Mediterranean.[d]
Recent decades	Used as raw materials for many food products in Japan.[e] Hydrocolloids extracted from seaweed used in pharmaceutical, cosmetic and food industries in European and Western countries.[f]
1970s	Research into use of seaweeds as an indirect source of fuel.[b]
Recent years	Used as functional food ingredients in Western countries.[g]

[a]Venugopal (2008).
[b]FAO (2003).
[c]Chennubhotla et al. (2013).
[d]Guiry & Guiry (2014).
[e]Jaspars & Folmer (2013).
[f]Jiménez-Escrig (2011).
[g]Gómez-Ordóñez et al. (2010).

Table 1.4. Main uses of green, red and brown seaweeds.

Seaweed	Component	Main uses
Green[a]	Rare cell wall polysaccharides (ulvan)	Dietary fibre with prebiotic potential
Red[b]	Hydrocolloids (agar and carrageenan)	Cosmetics, food preparations and biomedical and biotechnology research
Brown[c]	Hydrocolloids (alginate)	Emulsifiers, anticoagulants, food products, cosmetics, luxury spa items, fertilisers, textiles and rubbers
Other constituents[defg]	Minerals	Food supplements
	Pigments (general)	Natural dyes, antioxidants and vitamins
	Chlorophyll	Food and pharmaceutical colourants
	Phycobiliproteins	Dyes in food and cosmetics fluorescent probes
	Carotenoids	Dietary sources to meet nutritional requirements

[a]Ale et al. (2012).
[b]Morrissey et al. (2001).
[c]FAO (2003).
[d]Venugopal (2008).
[e]Sousa et al. (2008).
[f]Tierney et al. (2010).
[g]Fiedor & Burda (2014).

1.3.1 Colloids extracted from seaweeds

Following utilisation of seaweeds as an edible foodstuff, the second largest use of harvested seaweed is colloid extraction. Agar, carrageenan and alginates (seaweed polysaccharides), collectively referred to as phycocolloids, hydrocolloids or gums, function as food fibres, gelling and thickening agents which have led to a number of applications in food technology (Rupérez, 2002). The major producer of seaweeds for the extraction of hydrocolloids is China, followed by Japan and Korea (Werner et al., 2004). The manufacturing of colloids is concentrated in a few developed nations: Denmark, France, Japan, Norway, Spain, the United Kingdom and the United States. Colloid manufacture is slowly developing in a few seaweed producing countries in Asia (Marine Foresight series, 2005).

Seaweed hydrocolloids are commercially used in a variety of foods including bakery, confectionery, dairy products and muscle foods providing thickening and texture to foods. As thickeners, seaweed hydrocolloids are used in soups, gravies, salad dressings, sauces and toppings while as gelling agents, they are extensively used in products like jam, jelly, marmalade, restructured foods and low sugar/calorie gels (Saha & Bhattacharya, 2010). Other uses in various industrial sectors include textile, medicine and biotechnology applications (Venugopal, 2008).

To date, extensive research has explored the use of colloids from seaweeds in muscle based foods. Several hydrocolloids including carrageenan are typically used to improve the mechanical properties of processed fish products such as surimi gels (Ramírez et al., 2011). Addition of seaweed hydrocolloids into muscle foods has improved shelf-life and organoleptic properties of restructured meat products. Carrageenan and alginates have been used in meat for their water binding properties, influence on water holding capacity and functionality in muscle foods (Werner et al.,

2004). The texture of reformulated meat products can be altered and binding properties can be improved by the gel-forming properties of calcium alginate (Jiménez-Colmenero et al., 2001).

1.4 Biodiversity and ecology of seaweeds

Algae are divided into two groups: microalgae (microscopic and often unicellular) and macroalgae (multicellular organisms) (Usov & Zelinsky, 2013). Macroalgae, known as seaweeds, are autotrophic, aerobic organisms that generally live attached to rock or other hard substrata in coastal areas. A variety of abiotic and biotic factors determine the zonation and growth of different seaweed species. Important abiotic factors include: light regime (intensity, spectral composition and day length), followed by temperature, salinity, nutrient availability, and available substrate and wave exposure. Competition for substrate, grazing and epigrowth are considered the most important biotic factors (Hallerud, 2014). Seaweeds can reproduce in a variety of ways (vegetative, asexual and sexual) and differ significantly to terrestrial plants. For example, seaweeds do not possess flowers; therefore traditional methods of cross-pollination are not available to them (Werner et al., 2004).

1.5 Classification of seaweeds

Seaweed classification is based on the three different groups of light harvesting and photoprotective pigments: chlorophylls, phycobiliproteins and carotenoids. All seaweeds contain the light-harvesting pigment chlorophyll *a* (Hallerud, 2014). Seaweeds are commonly classified into three main groups: green (phylum Chlorophyta), dominated by chlorophyll *a* and *b*; red (phylum Rhodophyta), as a result of phycoerythrin and

phycocyanin; and brown (class Phaeophyceae), due to the presence of fucoxanthin (Rindi et al., 2012; Tierney et al., 2010).

1.5.1 Green seaweed

Chlorophyta are generally small, ranging from a few centimetres to a metre in length. Green seaweeds contain a wide variety of morphologies, ranging from unicells and filaments to blades and fleshy thalloid forms (FAO, 2013). Green seaweeds are less common in the ocean (salt waters) than red and brown seaweeds and often reside in areas with abundant light such as shallow water and tide pools forming symbiotic relationships with protozoa, sponges and coelenterates (Venugopal, 2011).

1.5.2 Red seaweed

Red seaweeds are similar in size to green seaweeds and range from red to purple and sometimes brownish red but are still classified as Rhodophyta because of their characteristics. Most red seaweeds are found in salt waters from low tide marks to greater depths up to 100 metres beneath the surface of the sea (Bhakuni & Rawat, 2005). Red seaweeds reside in a variety of locations such as the cold waters of Nova Scotia and southern Chile, as well as more temperate waters like the coasts of Morocco, Portugal and in tropical waters like Indonesia and the Philippines (FAO, 2003).

1.5.3 Brown seaweed

Brown seaweeds are extremely variable in shape and range from 30-60 centimetres to thick leather-like seaweed around 2-4 metres to giant kelp up to 20 metres in length (Venugopal, 2008). Phaeophyceae have very flexible stems and are found primarily in marine shallow waters or on shoreline rocks (Morrissey et al., 2001). Brown

seaweeds grow in both the Northern and Southern Hemispheres in cold waters, thriving best in waters up to about 20°C but can be found in warmer waters also (FAO, 2013).

1.6 Composition of seaweeds

Comprehensive reviews on seaweed composition have been reported by Chandini et al. (2008) and MacArtain et al. (2007). In general, the composition of fresh seaweed is approximately 87% water and 13% dry matter (DM) (Bhattacharjee & Islam, 2014). Seaweeds are considered a low caloric food, composed of polysaccharides, lipids, protein, vitamins, minerals and pigments.

1.6.1 Polysaccharides

Structural components of seaweeds differ greatly to those of terrestrial plants (e.g. cellulose, hemi-cellulose and lignin) (Anastasakis et al., 2011). Polysaccharides (4-76% DM) are a major constituent of seaweeds (Laurie-Eve Rioux et al., 2010). Seaweed polysaccharides include: alginate, carrageenan, agar, laminarin, fucoidan, ulvan, floridean starch and cellulose. Polysaccharides are characterised by sugar residues and the nature of the bond between them (Elleuch et al., 2011). Seaweed polysaccharides are divided into three groups based on their role: structural polysaccharides, intercellular mucilage and storage polysaccharides (Kim, 2012). A summary of polysaccharides present in green, red and brown seaweeds is presented in Table 1.5.

1.6.1.1 Green seaweed polysaccharides

Green seaweed contain cellulose like other vascular plants as the principle structural polysaccharide (Kim, 2012). Ulvan (8-29% DM), the main sulphated

Table 1.5. Structural polysaccharides, storage polysaccharides and intercellular mucilage of green, red and brown seaweeds.

Phylum/Class	Structural polysaccharide	Storage polysaccharide	Intercellular mucilage
Chlorophyta (green)	Cellulose[ab] Ulvan[bd] Glucuronan[b] Xylan[d] Mannan[d]	Amylose[ab] Amylopectin[ab]	Glucuronoxylorhamnans[a] Glucuronoxylorhamnogalactans[a] Xyloarabinogalactans[a]
Rhodophyta (red)	Cellulose[a] Agar[ad] Carrageenan[b]	Floridean starch[cb]	Agar[a] Carrageenan[b]
Phaeophyceae (brown)	Cellulose[a] Alginate[b]	Laminarin[bc]	Alginate[a] Fucoidan[ac] Laminarin[ac]

[a]Kim (2012).
[b]Chandini et al. (2008).
[c]Usov et al. (2001).
[d]Percival (1979).

polysaccharide of green seaweeds, is water soluble and composed of xyloglucan, glucuronan and cellulose in a linear arrangement (Vera et al., 2011). Ulvan is responsible for the maintenance of the osmolar stability and protection of the cell (Cardoso et al., 2015).

1.6.1.2 Red seaweed polysaccharides

Floridean starch (up to 35% DM), which differs from the starch of terrestrial plants by the absence of linear amylose, is the main storage polysaccharide present in red seaweeds (Usov & Zelinsky, 2013). Carrageenans (30-75% DM), agar (7-36% DM) and sulphated galactans are the main components of red seaweed cell walls (Vera et al., 2011). There are three major types of carrageenans based on their chemical composition and structure: kappa (κ), iota(ι) and lambda (λ) (Venugopal, 2008). Agar consists of at least two polysaccharides: agarose and agaropectin (Cardoso et al., 2015).

1.6.1.3 Brown seaweed polysaccharides

Brown seaweeds contain the largest amount of polysaccharides (over 50% DM), most of which are sulphated (Kim, 2012). The main structural components of brown seaweeds are alginic acid, mannitol, laminarin and fucoidan (Rupérez & Saura-Calixto, 2001). Alginic acid (10-45% DM) is partially responsible for flexibility of the seaweed (Vera et al., 2011). The main food reserves present in brown algae are laminarin (2–35% DM) and mannitol (5–25% DM). Mannitol appears to be the primary product of photosynthesis and sometimes is found attached to laminarin chains (Devillé et al., 2004). Fucans or fucoidan (5–20% DM) are sulphated polysaccharides that protect seaweed from desiccation (Anastasakis et al., 2011). A summary of the polysaccharides and sugars associated with green, red and brown seaweeds are presented in Table 1.6.

1.6.2 Lipids

Seaweed lipids consist of phospholipids (10-20% total lipid content), glycolipids (31-56% total lipid content) (glycosylglycerides) and non-polar glycerolipids (neutral lipids) (Kumari et al., 2013). Sterol compositions vary amongst seaweeds with cholesterol and fucosterol being the dominant sterol of red and brown seaweeds, respectively. The predominant sterol of green seaweeds varies amongst orders (i.e. isofucosterol the predominant sterol of the order Ulvales). Although seaweed lipid content is low (usually 1-5% DM), it is a rich source of polyunsaturated fatty acid (PUFA) containing 18 (C18) and 20 (C20) carbons, which is of great interest to the food industry (Chandini et al., 2008). In general, the PUFA content (10-70% of total fatty acids) of seaweeds is equal to or higher than terrestrial plants and naturally exists in a nutritionally ideal ω-6/ ω-3 (*n*-6/ *n*-3) free fatty acid ratio (Venugopal, 2008).

Table 1.6. Polysaccharide composition of green, red and brown seaweeds.

Phylum/Class	Polysaccharide	Main chain	Sugars
Chlorophyta	Cellulose[e]	linear β-(1,4)-D-glucan	
(green)	Ulvan[ac]	linear β-(1,4)-D-xyloglucan, glucuronan and cellulose	rhamnose, xylose, glucose, uronic acid, glucuronic acid, iduronic acid
Rhodophyta	Cellulose[e]	linear β-(1,4)-D-glucan	
(red)	Carrageenan[df]	linear α-(1,3)-D-galactose and β-(1,4)-D-3,6-anhydrogalactose	galactose, 3,6-anhydrogalactose, glucose
	Agar[dg]	linear α-(1,4)-D-galactose and α-L-3,6-anhydrogalactose	galactose, 3,6-anhydrogalactose, glucuronic acid
	Xylan[ah]	linear β-(1,4)-D-xylopyranose interspersed by single β-(1,3)-D-xylopyranose	mannose
	Floridean starch[a]	α-(1,4)-D-glucopyranose with numerous α-(1,6)-glucosidic branches	
Phaeophyceae	Cellulose[e]	linear β-(1,4)-D-glucan	
(brown)	Laminarin[afg]	linear β-(1,3)-D-glucopyranose	glucose and mannose
	Fucoidan[acg]	sulphated α-(1,3)-L-fucopyranose	fucose, galactose, xylose, glucuronic acid
	Alginate[abc]	β-D-mannuronic and α-L-guluronic acids with β-(1,4) linkages	

[a]Usov & Zelinsky (2013).
[b]Kim (2012).
[c]Cardoso et al. (2015).
[d]Renn (1997).
[e]Mišurcová et al. (2012).
[f]Gómez-Ordóñez, et al. (2010).
[g]Rupérez & Saura-Calixto (2001).
[h]Lahaye & Kaeffer (1997).

Green seaweeds are rich in alpha linolenic acid (C18:3 (*n*-3)) and the presence of docosahexaenoic acid (DHA) is reported for species in *Ulva* (Tierney et al., 2010). Red and brown seaweeds are rich in fatty acids with 20 carbon atoms such as eicosapentanoic acid (C20:5 (*n*-3)) (EPA) and arachidonic acid (C20:4 (*n*-6)) (Burtin, 2003). Brown

seaweeds contain primarily C16, C18 and C20 fatty acids with 10-15% of the lipid content made up of palmitic acid (C16:0) (Kim, 2012). Seaweeds are believed to be the only plant source of EPA and DHA (Rasmussen & Morrissey, 2007).

1.6.3 Protein

The protein content of seaweeds varies largely between species. Several reviews on seaweed proteins and peptides have been published previously (Fleurence, 1999; Harnedy & FitzGerald, 2011). Highest protein contents are reported for green and red seaweeds (averaging 10-30% DM) while brown seaweeds in general have lower protein contents (5-15% DM). *Ulva spp.* (green seaweeds) protein levels range 15-20% DM. In red seaweeds such as *Palmaria palmata* and *Porphyra tenera*, protein content can represent up to 35 and 47% DM, respectively, which is comparable to high protein vegetables like soybeans (35% DM) (Burtin, 2003). Seaweed proteins are complex bio macromolecules that are likely to exist as large protein or protein/pigment complexes. Their functional properties, e.g., gelation and emulsifying capacity, are comparable with those of terrestrial plants (Phillips & Williams, 2011).

The nutritional value of proteins depends on their essential amino acid composition and digestibility (Friedman, 1996). Seaweed proteins contain all the essential amino acids at levels sufficient to meet dietary requirements of humans but vary between species (Dawczynski et al., 2007). Alanine, aspartic and glutamic acid are the most abundant amino acids in seaweeds (Venugopal, 2008). Aspartic and glutamic acids make up 26-32% of green seaweed amino acids, 22-44% of brown seaweed amino acids but less than 20% of red seaweed amino acids (Fleurence, 1999). Taurine is particularly abundant in red and brown seaweeds (Jaspars & Folmer, 2013). Tryptophan is the first limiting amino acid (levels are insufficient to meet nutritional requirements for human

health) in seaweeds. Other amino acids present at low concentrations are leucine and isoleucine in red seaweeds and methionine, cysteine and lysine in brown seaweeds (Wong & Cheung, 2001).

The digestibility of seaweed protein varies according to species, environmental factors and the presence of anti-nutritional factors such as polyphenols, polysaccharides and protease inhibitors (Galland-Irmouli et al., 1999). According to some studies, the *in vitro* digestibility of seaweed protein relative to casein (100%) ranges from 56 to 67% in the presence of pancreatin. Wong & Cheung (2000) compared the *in vitro* digestibility of protein concentrates from red (*Hypnea charoides* and *Hypnea japonica*) and green (*Ulva lactuca*) seaweeds. The values ranged from 85.7 to 88.9% relative to casein, with red seaweed protein isolates having a slightly higher digestibility. Inclusion of the digestive enzyme, pronase, can increase the digestibility of seaweed protein to 78-95%, however the presence of polysaccharides and their interactions with proteins may reduce the accessibility of proteins to proteolysis (Fleurence, 1999). Galland-Irmouli et al. (1999) reported that the low digestibility of seaweed protein was most likely due to the presence of xylan (the main water soluble polysaccharide in *P. palmata*) which inhibited proteolytic digestive enzymes from proteolysis during digestion.

1.6.4 Vitamins

Seaweeds are an excellent source of vitamins (A, B_1, B_2, B_3, B_5, B_9, C, D, E and K) and one of the few vegetable sources of vitamin B_{12} that is structurally similar to animal sources (Jaspars & Folmer, 2013; Kraan & Dominguez, 2013). In general, green and red seaweed have higher contents of B vitamins than brown seaweed (Morgan et al., 1980). Vitamin C of green and brown seaweeds average between 500 to 3000 mg/kg DM while red seaweeds contains about 100 to 800 mg/kg (Burtin, 2003). The highest levels

of vitamin C are reported from spring to early summer (Kim, 2012). Most red and green seaweeds contain large amounts of vitamins B_1, B_2 and B_{12} as well as significant quantities of provitamin A (Kraan & Dominguez, 2013). Brown seaweeds contain the highest levels of vitamin E including alpha, beta and gamma tocopherol while green and red seaweeds only contain alpha tocopherol (Burtin, 2003).

1.6.5 Minerals

Seaweeds have exceptionally high ash contents, in some species, the mineral content can account for up to 36-50% DM (Burtin, 2003). By comparison, the mineral content of land vegetation accounts for only 5-10% DM (Kim, 2012). In general seaweeds are a rich source of iron, potassium, magnesium, calcium, sodium and phosphorous (Jaspars & Folmer, 2013). Compared to terrestrial plants, seaweeds are particularly rich in iodine and selenium. Additionally, seaweeds are one of the highest vegetable sources of calcium, with as much as 7% DM in some species and 25-34% DM in calcified seaweeds (Kraan & Dominguez, 2013).

1.6.6 Pigments

Photosynthetic pigments are used by seaweeds during photosynthesis to capture solar energy. There are three major categories of photosynthetic pigments: chlorophylls, carotenoids and phycobiliproteins (Rasmussen & Morrissey, 2007). According to their chemical structure, pigments are classified into the following major categories: closed tetrapyrroles (chlorophyll a and b), porphyrins (chlorophyll c), open tetrapyrrols (phycobilipigments) and polyisoprenoids with terminal cyclohexane rings (carotenoids – carotenes and xanthophylls). The general photosynthetic pigments for green, red and brown seaweeds are presented in Table 1.7.

Table 1.7. Colour pigments of green, red and brown seaweeds.

Phylum/Class	Chlorophyll	Phycobiliproteins	Carotenes	Xanthophylls
Chlorophyta (green)[c]	*a* and *b*	n/a	α and β-carotene	Lutein Zeaxanthin Antheraxanthin Neoxanthin Violaxanthin
Rhodophyta (red)[bc]	*a*, *b* and *d*	phycoerythrin, phycocyanin allophycocyanin	α and β-carotene	Lutein Zeaxanthin
Phaeophyceae (brown)[ac]	*a* and *c*	n/a	β-carotene	Lutein Fucoxanthin Violaxanthin

[a]Haugan & Liaaen-Jensen (1994).
[b]Schubert et al. (2006).
[c]Stengel et al. (2011).

1.6.6.1 Chlorophylls

Green pigments found in all seaweeds are lipid soluble chlorophylls which are structurally composed of two parts: a substituted porphyrin ring with a centrally bound magnesium atom and diterpene alcohol (phytol-long carbon chain). Chlorophyll *a* found in all seaweeds is an essential component of photosynthesis. Three other types of chlorophyll exist: *b*, *c* and *d* (Kraan & Dominguez, 2013). The diversity of chlorophyll is significant due to the natural abundance of chlorophyll present in seaweed. Several distinct derivatives of chlorophyll exist through processing and preparation of seaweed due to the fact chlorophyll is sensitive to extreme pH and temperature changes (Pangestuti & Kim, 2011).

1.6.6.2 Phycobiliproteins

Phycobiliproteins are water soluble protein-pigment complexes generally representing 1-10% DM of red seaweeds (Rasmussen & Morrissey, 2007). Phycobiliproteins are oligomeric proteins built up from chromophore-bearing polypeptides which are organised in supramolecular complexes called phycobilisomes

(Smit, 2004). In red seaweeds, phycobiliproteins play an important role in utilising light in deeper waters during photosynthesis (Kraan & Dominguez, 2013). The covalently bound prosthetic groups (open chain tetrapyrrole chromophores bearing A, B, C and D rings named phycobilins) determine the colour of the phycobiliproteins (Eriksen, 2008). The four colours are blue, red, yellow or purple due to phycocyanobilin, phycoerythrobilin, phycourobilin or phycobiliviolin, respectively (Sekar & Chandramohan, 2008). The four main classes of phycobiliproteins in red seaweeds include: phycocyanin (blue), allophycocyanin (bluish-green), phycoerythrocyanin (orange) and phycoerythrin (purple) (Sidler, 2004). Phycoerythrins are the most abundant phycobiliproteins found in red seaweed (Pangestuti & Kim, 2011).

1.6.6.3 Carotenoids

Carotenoids (terpenoids) are mostly polyunsaturated hydrocarbons containing 40 carbon atoms and two terminal ring systems responsible for the red, orange and yellow colours found in seaweed (Tierney et al., 2010). Carotenoids are considered accessory pigments and function both as light energy harvesters and as antioxidants to protect seaweeds from reactive oxygen species (ROS) formed by exposure to light and air (Rasmussen & Morrissey, 2007). The most researched function of carotenoids is their provitamin A activity, especially that of β-carotene and to a lesser extent of lutein (Kiokias & Oreopoulou, 2006).

Carotenoids are lipid soluble and are divided into two groups: carotenes (hydrocarbons) and xanthophylls (containing oxygen) (Pangestuti & Kim, 2011). All seaweeds are able to synthesize lycopene, a precursor for the two different synthesis pathways of carotenoids (β,ε-carotene and β,β-carotene) (Schubert et al., 2006). β-carotene is a component of the photosynthetic reaction centre which protects the

organism from damage during excessive irradiances by preventing the formation of ROS (Khattar & Kaur, 2009). Typically carotenes are more orange in colour than xanthophylls.

Xanthophylls (yellow pigments) are oxidation products of carotenes and vary considerably according to light environment. The diversification of xanthophylls increases by the inclusion of allene or acetylene groups (Schubert et al., 2006). Fucoxanthin (xanthophyll) is the major carotenoid present in brown seaweed and accounts for more than 10% of the estimated total amount of carotenoids in nature (Tierney et al., 2010). When seaweeds are exposed to excess light conditions, the xanthophyll cycle (found in green and brown seaweeds and sometimes red seaweeds) is a mechanism used to protect against photo oxidative damage (Schubert et al., 2006). Essentially seaweeds synthesize antheraxanthin and zeaxanthin from existing violaxanthin by a rapid two step de-epoxidation process. Zeaxanthin functions to protect the seaweed from oxidative damage while antheraxanthin and violaxanthin serve as light energy harvesters (Hallerud, 2014).

1.6.7 Factors influencing seaweed composition

The chemical composition of seaweeds varies considerably due to a number of factors including harvesting region, season and seaweed species. Polysaccharide content varies greatly between seasons due to the higher rates of photosynthesis during brighter months leading to a build up of polysaccharides in seaweeds (Hallerud, 2014). Conversely, seaweeds contain a higher amount of protein in winter, while protein levels are lower during the summer months, influenced by environmental factors such as light, temperature and salinity (Kim, 2012). The mineral content of seaweeds varies with species, growing region, season and environmental conditions. Mineral levels reach

maximum concentrations in specimens habituated in areas where rivers drain into the sea from mineralized areas (Kim, 2012).

Seaweeds possess an efficient antioxidant defence system as a result of living in a harsh environment (Chandini et al., 2008). The defence system of seaweeds includes many forms of antioxidant compounds such as vitamins and pigments, which afford protection against direct sunlight in an aqueous environment, therefore seaweeds growing in the littoral zone or on the surface will have higher concentrations of these antioxidant compounds (Kraan & Dominguez, 2013). In addition, pigment content can be influenced by polysaccharide composition. High amounts of polysaccharides may lead to reduced levels of pigments (Hallerud, 2014). Environmental changes such as the quality of light can also have a profound effect on the colour pigments present in the seaweed (Dawson, 2007).

1.7 Bioactive compounds in seaweeds

Screening seaweeds for bioactive constituents for biomedical and pharmaceutical applications has been the focus of many studies for the last decade. Bioactivity is the specific effect upon exposure to a substance and includes tissue uptake and the consequent physiological response (such as antioxidant or anti-inflammatory) (Carbonell-Capella et al., 2014). Seaweeds contain a tremendous diversity of bioactive compounds (polysaccharides, fatty acids, peptides, carotenoids, phenols, minerals, sulphur compounds, vitamins, etc.) with structures different to those found in terrestrial plants (Gupta & Abu-Ghannam, 2011a; Jiménez-Escrig et al., 2011). Differences in chemical composition and biological activities of seaweeds due to natural variability have been reviewed by Stengel et al. (2011). The proliferation period (winter to spring) has been suggested as the optimal harvest period of seaweeds to maximise the quantity of

biologically active components available (Kim, 2012). This may be a result of cold stress which elevates levels of ROS in seaweeds during the winter months (Cornish & Garbary, 2010).

Seaweed extracts and purified fractions from seaweeds with antioxidant, antimicrobial, anti-tumor, anti-coagulant and antiviral activities have been extensively researched and reviewed (Gamal-Eldeen, 2009; Gupta & Abu-Ghannam, 2011b; Wijesekara et al., 2011). Seaweeds represent one of the richest sources of natural antioxidants and antimicrobial substances amongst marine organisms as they have evolved to protect themselves against oxidative stress from ultra-violet radiation, desiccation and extreme temperature fluctuations at low tide (Percival, 1979). Antioxidant activity of seaweeds and seaweed extracts will be the main focus in terms of bioactivity for the remainder of this review. Hydrophilic (polyphenols, phycobiliproteins and vitamins) and lipophilic (carotenoids and α-tocopherol) antioxidants isolated from seaweed have been extensively reviewed (Chandini et al., 2008; Eriksen, 2008; Kraan & Dominguez, 2013; Plaza et al., 2008; Prasanna et al., 2007; Sousa et al., 2008; Vadlapudi, 2012; Yuan & Walsh, 2006). Collectively, previously reported literature suggests that seaweed and seaweed constituents such as sulphated polysaccharides may prove to be a useful source of effective, non-toxic antioxidants for use in the food industry (Tierney et al., 2010).

1.8 Methodologies for measurement of antioxidant bioactivity

Antioxidants (endogenous enzymes and dietary antioxidants) protect the human body against reactive oxygen and nitrogen species (ROS and RNS) which can attack and cause damage to membrane lipids, proteins and DNA resulting in a variety of health disorders such as cancer and diabetes mellitus (Fiedor & Burda, 2014). Antioxidants

have different modes of action to prevent damage caused by oxidative stress including: radical chain reaction inhibitors, metal chelators, oxidative enzyme inhibitors and antioxidant enzyme cofactors (Moure et al., 2001). As a result of the different mechanisms of action exhibited by antioxidants, many methods to ascertain antioxidant capacity of compounds have been developed and utilised. Methodologies differ from one another in terms of reaction mechanisms, oxidant and target/probe species, reaction conditions and expression of results (Karadag et al., 2009). Assays have been developed to test the antioxidant potential of seaweed compounds *in vitro* and *in vivo* using chemical reaction assays, cell culture models and animal feeding trials.

1.8.1 *In vitro* chemical reaction antioxidant assays

Approximately 20 analytical methods exist to determine antioxidant capacity of seaweeds/seaweed extracts involving different reagents, reaction mixture composition and standards etc. A comparison of results is often difficult owing to the array of methods employed under a variety of experimental conditions as well as differences in the physicochemical properties of oxidisable substrates (Karadag et al., 2009). Chemical reaction based assays are excellent preliminary tools, however no single assay can be considered as the benchmark for the assessment of antioxidant capacity as each assay has limitations (Tierney et al., 2010).

Commonly used *in vitro* antioxidant assays are divided into (1) hydrogen atom transfer (HAT) reaction-based assays and (2) the electron transfer (ET) reaction-based assays (Karadag et al., 2009). HAT and ET reaction-based assay usually employ a substrate which undergoes a colour change upon reaction thereby allowing antioxidant activity to be measured spectrophotometrically (Huang et al., 2005). HAT reaction-based assays measure the ability of an antioxidant to scavenge free radicals by donating a

hydrogen atom to form stable compounds and are more relevant to the radical chain breaking antioxidant capacity (Prior, 2004). ET reaction-based methods detect the ability of a potential antioxidant to transfer one electron to reduce any compound which includes metals, carbonyls and radicals (Karadag et al., 2009).

(1) $AH + X\bullet \rightarrow XH + A\bullet$

(2) $M(III) + AH \rightarrow AH\bullet + M(II)$

The most commonly used ET reaction-based assays include: total phenol content (TPC), ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging and the Trolox equivalent antioxidant capacity (TEAC). HAT reaction-based assays employed in measuring antioxidant activity of seaweeds are oxygen radical absorbance capacity (ORAC), β-carotene bleaching assay (BCBA), total radical trapping antioxidant parameter (TRAP) and total oxidant scavenging capacity (TOSC) (Karadag et al., 2009; MacDonald-Wicks et al., 2006). Methodologies for determining *in vitro* antioxidant activity/capacity are well reviewed by MacDonald-Wicks et al. (2006). Balboa et al. (2013) has extensively reviewed the use of chemical reaction assays to determine the antioxidant activity of brown seaweeds.

1.8.1.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

For the purposes of this review the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay will be discussed further. The DPPH free radical scavenging assay is one of the oldest methods used to assess antioxidant activity of seaweed extracts (Huang et al., 2005). The DPPH radical is well known organic nitrogen radical which is deep purple in colour. The purple chromogen radical is reduced by antioxidant/reducing compounds to a corresponding pale yellow hydrazine (Karadag et al., 2009). Originally the reaction was monitored by electron paramagnetic resonance (ESR) spectroscopy and

relied on the signal intensity of the DPPH radical being inversely related to the concentration of the antioxidant and the reaction time. More frequently, however, the reaction is monitored by a timed decoloration assay where the decrease in absorbance at 515-528 nm produced by the addition of the antioxidant to the DPPH radical in methanol or ethanol is measured (MacDonald-Wicks et al., 2006). The DPPH free radical scavenging activity may be expressed as either the % DPPH inhibition or as a half maximal effective concentration (EC_{50}) (concentration required to obtain a 50% antioxidant effect) (Karadag et al., 2009).

There are some limitations of the DPPH free radical scavenging assay. DPPH can only be dissolved in organic solvents which is an important factor when interpreting the role of hydrophilic antioxidants. Furthermore the DPPH radical has little similarity to the highly reactive and transient peroxyl radicals formed during the breakdown of organic molecules (Karadag et al., 2009). Additionally, antioxidants which react quickly with peroxyl radicals *in vivo* may react slowly or even be inert to the DPPH radical (Huang et al., 2005). Another consideration for this assay is the reaction time with the DPPH radical. The reaction kinetics between the DPPH radical and the antioxidant is not linear to the concentration of the DPPH radical, therefore expressing results as EC_{50} can be difficult. The rate of reaction can be increased or decreased by basic and acidic impurities, respectively (MacDonald-Wicks et al., 2006). Lastly, interpretation of the results can be complicated if the compounds with antioxidant activity have a spectra that overlap at 515 nm (Karadag et al., 2009).

DPPH free radical scavenging activity of seaweed extracts has been reported for a number of seaweed species (Machová & Bystrický, 2013; Mak et al., 2013). The ability of seaweed extracts to quench DPPH radicals is known to take place over a longer period

of time than rapid acting synthetic antioxidants such as butylated hydroxyanisole (BHA) (Abu-Ghannam & Cox, 2014; Yuan et al., 2005).

1.8.2 *In vivo* antioxidant capacity assays

Endogenous enzymes such as superoxide dismutase (SOD), glutathione peroxidise (GSHPx), catalase (CAT), glutathione (GSH) as well as dietary antioxidants like α-tocopherol, α-carotene and ascorbic acid play a role in the antioxidant defence system (Ngo et al., 2011). When host antioxidant defences are overwhelmed by the presence of excessive quantities of free radicals, oxidative stress occurs resulting in damage to various biological components (i.e. lipids, proteins and DNA) (Wood et al., 2006). Kohen and Nyska (2002) present a comprehensive review of oxidation in biological systems. The variety of direct and indirect methods commonly used to measure *in vivo* antioxidant activity of living systems (i.e. animals and humans) is well reviewed by Wood et al. (2006).

1.8.2.1 Direct markers of antioxidant activity

Direct markers of antioxidant activity include measuring levels of dietary antioxidants as well as endogenous antioxidants. A summary of direct markers of antioxidant activity reported by Wood et al. (2006) is presented in Table 1.8. Measurement of antioxidant levels is useful to confirm the uptake of a particular compound, however interpretation of such data is limited as a delicate balance exists between various compounds which make up the antioxidant defence system (Kohen & Nyska, 2002). Generally dietary antioxidants are measured using analytical techniques such as high-performance liquid chromatography (HPLC) while endogenous antioxidants are analysed by a spectrophotometric assays (Wood et al., 2006)

Using various antioxidant/reducing assays such as ORAC, TRAP, FRAP and TEAC is a direct method to measure total antioxidant capacity (TAS) of blood plasma/serum. Measuring blood plasma/serum TAS is particularly useful in evaluating the efficacy of extracts that are not well understood or characterised, for example plant extracts such as seaweeds (Kohen & Nyska, 2002; Wood et al., 2006).

Table 1.8. Direct markers of antioxidant activity[a].

Antioxidant concentrations		**Total antioxidant capacity**
Dietary	Endogenous	
Vitamins E, C	GSH	ORAC
Carotenoids	GSHPx	TRAP
Flavonoids	SOD	FRAP
	CAT	TEAC

[a]Wood et al. (2006).

1.8.2.2 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay

For the purposes of this review the 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) decolorization assay will be discussed briefly. ABTS assay is a method commonly employed to measure antioxidant capacity of both lipophilic and hydrophilic antioxidants using a spectrophotometer (Wood et al., 2006). When ABTS, a peroxidase substrate, is oxidized by peroxyl radicals or other oxidants in the presence of H_2O_2 the metastable radical cation $ABTS^{*+}$ is formed. $ABTS^{*+}$ is intensely blue/green coloured and can be monitored spectrophotometrically in the range of 600-750 nm (Karadag et al., 2009). The antioxidant capacity is measured as the ability of test compounds to decrease the colour reacting directly with $ABTS^{*+}$ radical and expressed relative to Trolox (Kohen & Nyska, 2002).

1.8.2.3 Indirect markers of antioxidant activity

Measuring the peroxidation products which result due to oxidative stress is an indirect way to assess antioxidant capacity. A summary of indirect markers of antioxidant activity reported by Wood et al. (2006) is presented in Table 1.9. Lipid, protein and DNA damage of various cells and organs (i.e. muscles, brain, liver, heart, kidney and plasma) harvested from animals following the desired supplementation period can be assessed using various assays (Kohen & Nyska, 2002).

Oxidative damage to lipids leads to the formation of numerous lipid peroxidation products such as F2-isoprostanes, 4-hydroxy-2-nonenal (4-HNE), malondialdehyde (MDA) and alkanes (ethane, pentane and isoprene) which can be measured as indices of oxidative stress (Kohen & Nyska, 2002). Isoprostanes are commonly quantified using gas chromatography-mass spectrometry (GC-MS) and enzyme immunoassay (EIA). 4-HNE is measured reliably using HPLC or enzyme-linked immunosorbent assay (ELISA) (Wood et al., 2006). MDA concentrations are usually determined spectrophotometrically by the thiobarbituric acid-reactive substances (TBARS) assay (Del Rio et al., 2005). Lipid hydroperoxides are measured using HPLC, GC-MS or commercial colorimetric assays. Hydrocarbons are generally measured in the exhaled breath using gas chromatography. Measurement of autoantibodies directed against oxidative modifications of low density lipoprotein (LDL) is usually determined by ELISA (Wood et al., 2006).

Oxidative damage to proteins can be measured by plasma protein carbonyl concentrations using atomic absorption spectroscopy, fluorescence spectroscopy or HPLC. Assays used to measure oxidative damage to specific amino acid residues in proteins include GC-MS and HPLC (Kohen & Nyska, 2002). The most commonly

measured product of DNA damage is 8-hydroxydeoxyguanosine (8-ohdG) using HPLC and ELISA kits (Wood et al., 2006).

Measuring free radicals is another indirect marker of antioxidant status as free radicals are quenched in the presence of antioxidants. Free radicals are measured using electron spin resonance spectroscopy (ESR), chemiluminescence and assays (colorimetric and flurometric) measuring the H_2O_2 radical (Kohen & Nyska, 2002; Wood et al., 2006).

Table 1.9. Indirect markers of antioxidant activity[a].

Markers of oxidative stress			**Free radical measurement**
Lipid damage	**Protein damage**	**DNA damage**	ESR
8-isoprostane	Protein carbonyls	8-ohdG	Chemiluminescence
TBARS/MDA	Nitrotyrosine		H_2O_2
4-HNE			
Lipid hydroperoxides			
Hydrocarbons			
Antibioties against LDL			

[a]Wood et al. (2006).

1.8.2.4 Thiobarbituric acid-reactive substances (TBARS) assay

For the purposes of this review the thiobarbituric acid-reactive substances (TBARS) assay will be discussed briefly. Malondialdehyde (MDA) is derivatised with 2-thiobarbituric acid (TBA) to form the MDA-TBA adduct, which is most commonly quantified using a spectrophotometric assay (Wood et al., 2006).

SECTION 2: SEAWEED SPECIFICS

For the purposes of this review *Ulva rigida, Palmaria palmata* and *Laminaria digitata* will be discussed further in sections 1.9-1.11.

1.9 Green seaweed (*Ulva rigida*) - Occurrence, habitat and structure

Ulva rigida
[a]Guiry & Guiry (2014).

Ulva species (*spp.*), commonly known as sea lettuce, are opportunistic fast growing seaweeds with two growing peaks in autumn and spring (Robic et al., 2009). Typically *Ulva spp.* are found in saline and salty waters, however they can also grow in freshwater habitats (Silva et al., 2013). *Ulva spp.* morphology resemble the typical shape of blade-shaped fronds, two cells thick with no tissue differentiation (Alves et al., 2013). Due to the morphological plasticity expressed by many members of the Family Ulvaceae, identification of *Ulva spp.* is difficult to classify and often research is executed using *Ulva spp.* rather than a specific species. There are about 100 species of *Ulva* currently identified by AlgaeBase even though nearly 600 names have been used in the past (Guiry & Guiry, 2014). Seven *Ulva spp.* exist on the shores of Ireland including: *Ulva rigida, U. scandinavica, U. lactuca, U. gigantea, U. rotundata, U. califonica* and *Umbraulva olivascens*.

Cell wall polysaccharides (38-54%) include two major: ulvan and cellulose as well as two minor: xyloglucan and glucuronan. Ulvan is a complex water soluble sulphated polysaccharide mainly composed of rhamnose, glucuronic acid, iduronic acid, xylose and sulphate (Lahaye & Robic, 2007). The solubility of ulvan may be enhanced through increased temperature (Alves et al., 2013).

1.9.1 Composition of *Ulva rigida*

Sea lettuce composition includes a significant amount of nutrients essential for the human body (Taylor, 2011). The compositional analysis of *U. rigida* is presented in Table 1.10. *U. rigida* is rich in minerals, balanced amino acid profiles, ascorbic acid (vitamin C) and significant levels of arginine, aspartic and glutamic acids (Norris et al., 1937; Ratana-arporn & Chirapart, 2006). Aspartic and glutamic acids are reported to be responsible for the characteristic flavour and taste of *U. rigida*. The sodium and potassium ratio of *U. rigida* is near to 1 (Taboada et al., 2010).

1.9.1.1 Colour pigments

Chlorophyll *a* (primary photosynthetic pigment) (13% DM) and chlorophyll *b* (accessory pigment) (7.5% DM) are found in *U. rigida*. The major carotenoids (4.5% DM) in *U. rigida* include β-carotene and yellow xanthophylls (lutein and zeaxanthin) (Satpati & Pal, 2011). The structures of the primary carotenoids found in *U. rigida* are presented in Figure 1.1. Briefly, β-carotene is structurally composed of an unsaturated hydrocarbon containing 40 carbon atoms per molecule terminated by a hydrocarbon ring, on both ends of the molecule (Krinsky et al., 2004; Scheer, 2013; Shahidi & Brown, 1998). The molecular structure of xanthophylls is similar to carotenes with one or more functional groups with oxygen being present as hydroxyl groups. Lutein and zeaxanthin

Table 1.10. Compositional analysis of *Ulva rigida*.

Macro/micronutrient	**%**
Water[x]	78-80%[c]
Protein[y]	15-25%[a]
Lipid[y]	0.6-1% [b]
Carbohydrate[y]	42-46%[b]
Ash[y]	13-22%[c]
Vitamin A	4286[b]
Vitamin C	100-200 ppm[b]
Vitamin B3	98 ppm[b]
Vitamin B12	6 ppm[b]
Calcium	7300 ppm[b]
Iodine	240 ppm[b]
Iron	870-1370 ppm[b]
Magnesium	2.8%[b]
Manganese	347 ppm[b]
Sodium	1.1%[b]
Potassium	0.7%[b]

[x]wet weight basis.
[y]dry weight basis.
[a]Burtin (2003).
[b]Morrissey et al. (2001).
[c]Guiry & Guiry (2014).

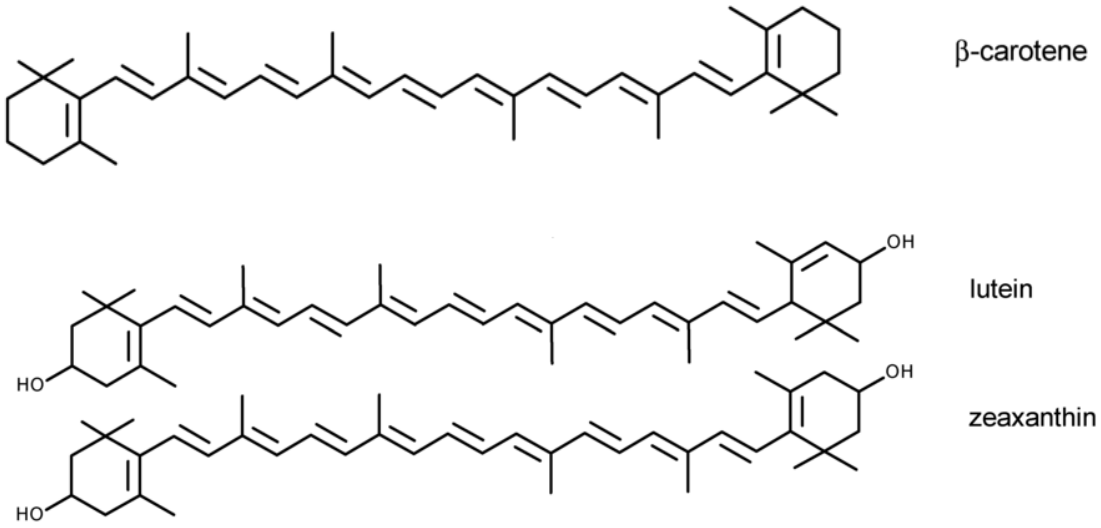


Figure 1.1. β-carotene (carotene), lutein and zeaxanthin (xanthophylls) structures present in *Ulva rigida*.
Source: Fiedor & Burda (2014).

are isomers and have identical chemical formulas with a 40 carbon long molecule and 11 conjugated double bonds. The only difference between the two xanthophylls is in the location of the double bond in one of the end rings. Lutein has one β-ring and one ε-ring, zeaxanthin has two β-rings. The position of the double bond in lutein forms a more chemically reactive allylic hydroxyl end group versus the extra conjugated double bond in zeaxanthin (Alves-Rodrigues & Shao, 2004; Breithaupt, 2007; Schubert et al., 2006). Zavodnik (1987) reported pronounced variation of pigments during the seasons with the highest level of chlorophylls and carotenoids during the initiation of growth (February to May). The carotenoid content is strongly correlated to chlorophyll content.

1.9.2 Bioactive compounds present in *Ulva rigida*

A few studies have evaluated crude extracts from *Ulva spp.* and *Ulva rigida* for antioxidant activity. The literature suggests the antioxidant activity of *U. rigida* is largely due to phenolic level; however other compounds present in *U. rigida* such as vitamin E, C and A are also linked to antioxidant activity (Yildiz et al., 2011). Farasat et al. (2013) reported the *in vitro* antioxidant activity (DPPH) of *Ulva spp.* was significantly correlated to the phenolic and flavonoid level. *In vitro* antioxidant activity of *U. rigida*, measured using DPPH assay, was reported highest in February and March compared to late summer and early autumn (August-October). The decrease of antioxidant activity is attributed to an increase of water temperature (> 20°C) and subsequent decline in total phenolic content. Lower temperature waters were positively correlated with an increase in antioxidant enzyme activity and photosynthetic capacity (Trigui et al., 2013).

1.9.2.1 Ulvan bioactivity

Despite having a host of health promoting effects, not many members of the genus Ulva have been extensively investigated for their biological properties. However the sulphated polysaccharide, ulvan, has attracted a lot of attention due to the different biological properties it possess as a result of the unusual chemical composition and structure. The structure and functional properties of ulvan has been reviewed by Lahaye & Robic (2007). In the recent years, ulvan has been investigated for use in the development of novel drugs and functional foods (Silva et al., 2013). Ulvan is linked to anti-fungal, antiviral, anti-coagulant, antithrombotic and immunomodulatory activities (Costa et al., 2010; Leiro et al., 2007; Wijesekara et al., 2011). Research demonstrates a strong correlation between degree of sulphation of ulvan and subsequent biological activities (Alves et al., 2013; Bocanegra et al., 2009).

Antioxidant activity of ulvan has been reported through scavenging activity of superoxide and hydroxyl radicals, metal chelating activity and reducing power (Costa et al., 2010; El Baky et al., 2009). The molecular weight of ulvan and its oligosaccharides, and sulphate content of ulvan and its derivatives influence antioxidant activity (Qi et al., 2005a; Qi et al., 2006; Qi et al., 2005b). The lowest molecular weight ulvan (28.2 kDa) demonstrated the strongest inhibitory effects on superoxide and hydroxyl radicals, as well as the strongest reducing power and metal chelating abilities. Chemical modification of ulvans can also enhance antioxidant activity. Qi et al. (2006) reported acetylated and benzoylated ulvans extracted from *U. pertusa* had enhanced *in vitro* antioxidant activity (scavenging activity, reducing power and chelating ability) compared to natural ulvan.

1.9.2.2 Pigment bioactivity

The antioxidant activity of the pigment chlorophyll and chlorophyll derivatives, related to structural features has been extensively reviewed by Pangestuti & Kim (2011). Hoshina et al. (1998) demonstrated a clear structural relationship within porphyrins for inhibition of lipid hydroperoxide formation. The presence and nature of the central metal as well as the porphyrin structure are considered important with respect to the antioxidant activity of chlorophyll derivatives. In seaweeds, carotenoids play the role of accessory pigments and photo protectors of oxidative damage. The same protective mechanisms of carotenoid pigments are recognised to play a major role in other tissues of living organisms, including humans (Sujak et al., 1999). Many reviews on lutein and zeaxanthin exist (Alves-Rodrigues & Shao, 2004; Sajilata et al., 2008). Lutein and zeaxanthin are the major carotenoids in the macular area of the human retina and are linked to eye health. Lutein and zeaxanthin protect against oxidative damage of the lipid matrix in macular membranes (Sujak et al., 1999). As a result, the carry-over of xanthophylls in the human food chain has been reported as advantageous for human health (Breithaupt, 2007).

1.9.2.3 Antimicrobial bioactivity

The antimicrobial activity of seaweeds is strongly linked to season as well as physical and biological factors like life cycle, herbivore pressure and light (Trigui et al., 2013). The literature suggests seaweeds defend themselves against bacterial fouling by the production of secondary metabolites which prevent the attachment or growth of bacterial colonizers (Cox et al., 2010). Seaweed compounds with reported antibacterial activity include amino acids, terpenoids, phlorotannins, acrylic acid, phenolic compounds, steroids, halogenated ketones and alkanes, cyclic polysulphides and fatty

acids (Chandrasekaran et al., 2014; Mtolera & Semesi, 1996; Nagaraj & Osborne, 2014; Niang & Hung, 1984). Limited information exists regarding the exact mechanism of antimicrobial action due to compounds extracted from seaweed. In general, these substances can attack the bacterial cell walls and membranes resulting in an extensive release of intercellular substances and/or disrupt the uptake and transport of substances by bacteria. Other modes of antimicrobial activity which have been attributed to seaweed compounds include reduction of protein and nucleic acid synthesis in the bacterial cell walls and the inhibition of bacterial cell respiration (Vatsos & Rebours, 2014).

Efficiency of the extraction method of bioactive compounds from seaweed also influences antimicrobial activity (Tüney et al., 2006). Commonly, water or organic solvents such as methanol, ethanol, acetone, ethyl ether, diethyl ether, ethyl acetate, chloroform, dichloromethane, benzyne, hexane, chloroform: methanol (2:1) and chloroform: ethanol (1:1, 2:1) are used to extract bioactive compounds from seaweeds (Bansemir et al., 2006). Conflicting evidence exists on whether extraction of bioactive substances is more effective from dry or fresh seaweed. Some studies reported that the test organisms were more sensitive to extracts of fresh seaweeds, while others show dried seaweed extracts exhibit broader and higher antibacterial activity (Kolanjinathan, 2011; Manilal et al., 2009; Tüney et al., 2006). The commonly employed *in vitro* antibacterial assays include: paper disc diffusion assay, absorbance measurements and growth studies (Qiao et al., 2010).

Antibacterial activity has been reported for *U. rigida* extracts against *Escheria coli* and *Pseudomonas aeruginosa*, however activity varied monthly with the highest inhibitory activity recorded during spring and summer (Trigui et al., 2013). Crude extracts (methanol 1:50 w/v) of *U. rigida* effectively inhibited the growth of *S. aureus* (Taskin et al., 2007). Diethyl ether extracts of *U. rigida* from the coasts of Urla, Izmir

demonstrated high antimicrobial activity (10-15-mm halo) against *Candida sp*., *E. faecalis, P. aeruginosa* and *E. coli* using the agar diffusion technique. Dried *U. rigida* extracts had significantly less antimicrobial activity compared to the fresh extract. The decrease in bioactivity was attributed to the loss of active materials during the drying process (Tüney et al., 2006).

1.10 Red seaweed (*Palmaria palmata*) - Occurrence, habitat and structure

Palmaria palmata
[a]Guiry & Guiry (2014).

Palmaria palmata, commonly known as dulse, dillisk, dilsk, and red dulse, is one of the more popular seaweed species for human consumption in the Western world (Rhatigan, 2009). Dulse is relatively small and grows attached to rocks or stipes of *Laminaria* and ranges in colour from deep rose to reddish-purple. The structure and composition of *P. palmata* cell wall polysaccharides has been extensively reviewed (Myers & Preston, 1959; Percival & Chanda, 1950). Linear xylans composed of mixed β-1,4 and β-1,3 linkages plus cellulose (2-7%) constitute most of *P. palmata* cell walls. Xylose is the major sugar with traces of mannose, galactose and glucose encompass sugar constituents of *P. palmata* (Myers & Preston, 1959). The soluble fibres are reported to have low viscosity, but high fermentability by colonic bacteria. The insoluble fibres of have equivalent hydration properties to those of land vegetables (Lahaye et al., 1993).

1.10.1 Composition of *Palmaria palmata*

The compositional analysis of *P. palmata* is presented in Table 1.11. The protein content (8-35% DM) of *P. palmata* has been reported as the second highest of all

seaweeds (Wang et al., 2010b). The most abundant amino acids are alanine, aspartic acid, glutamic acid and glycine. The large content of free glutamic acid and aspartic acid gives dulse a strong umami flavour (Mouritsen et al., 2013). *P. palmata* is far richer in potassium salts than in sodium salts compared to all other seaweeds with a K/Na ratio of approximately 4-5 (Morgan et al., 1980). The vitamin and mineral composition as well as nutritional value of *P. palmata* has been well reviewed (Morgan et al., 1980; Mouritsen et al., 2013). In comparison to terrestrial fruits and vegetables, *P. palmata* is a good source of iron, magnesium, calcium and iodine. Vitamin E (α-tocopherol) and vitamin C levels of *P. palmata* is highest in late summer and autumn. The carotene content varies throughout the year with no regular trend reported (Morgan et al., 1980).

Table 1.11. Compositional analysis of *Palmaria palmata*.

Macro/micronutrient	**%**
Water[x]	79-88%[c]
Protein[y]	12-21%[a]
Lipid[y]	0.7-3%[b]
Carbohydrate[y]	46-50%[b]
Ash[y]	15-30%[c]
Vitamin C	150-280 ppm[b]
Beta-carotene	663 i.u.[b]
Vitamin B1	7 ppm[b]
Vitamin B2	2-5 ppm[b]
Vitamin B3	2-19 ppm[b]
Vitamin B6	9 ppm[b]
Vitamin B12	6.6 ppb[b]
Vitamin E	1.71 ppm[b]
Calcium	2000-8000 ppm[b]
Iodine	150-550 ppm[b]
Iron	56-350 ppm[b]
Magnesium	0.2-0.5%[b]
Manganese	10-155 ppm[b]
Sodium	0.8-3%[b]
Zinc	3 ppm[b]

[x]wet weight basis.
[y]dry weight basis.
[a]Burtin (2003).
[b]Morrissey et al. (2001).
[c]Guiry & Guiry (2014).

1.10.1.1 Colour pigments

Chlorophyll *a* and *b* are found in *P. palmata* (Dawson, 2007). The phycobiliproteins of *P. palmata* include: R-phycocyanin (greenish blue), allophycocyanin (greenish blue), R-phycoerythrin (red) and *β*-phycoerythrin (red) (Prasanna et al., 2007). High light intensity and nitrogen deprivation are important factors in the loss of dark-red pigments (Morgan, et al., 1980). *P. palmata* also contains α and *β*-carotene and high levels of lutein (xanthophyll) (Chu, 2012). The structure of α-carotene found in *P. palmata* is presented in Figure 1.2, other structures of carotenoids are provided in section 1.9.1.1 Colour pigments. Briefly, α-carotene is structurally composed of an unsaturated hydrocarbon containing 40 carbon atoms per molecule terminated by a β-ionone ring at one end and an α-ionone ring at the other (Krinsky et al., 2004). Although zeaxanthin is reported for most Rodophyceae, it is totally absent in *P. palmata*. The carotenoid content is dependent on the postharvest treatment of the seaweed as well as light quality and intensity (Parjikolaei et al., 2013). Reported ratios of α-carotene / *β*-carotene ranges from 1.4 to 2.7 (Morgan et al., 1980).

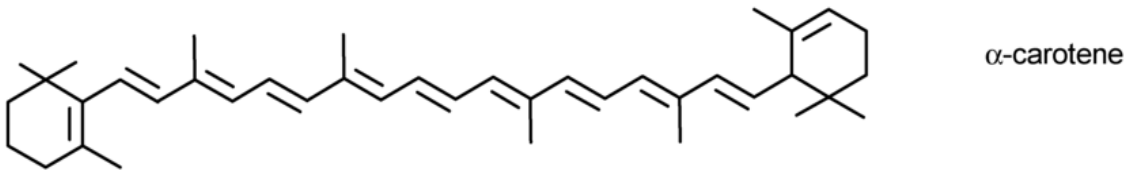


Figure 1.2. α-Carotene structure present in *Palmaria palmata*.
Source: Fiedor & Burda (2014).

1.10.2 Bioactive compounds present in *Palmaria palmata*

P. palmata contains several classes of hydrophilic antioxidant components including ascorbic acid, glutathione and polyphenols (Yuan et al., 2005; Yuan & Walsh, 2006). The antioxidant activity of a 1-butanol soluble fraction derived from methanol

extract of *P. palmata* was assessed comprehensively by a variety of *in vitro* methods (deoxyribose, DPPH, ABTS, TBARS). The *P. palmata* extract was active in scavenging OH radicals in the deoxyribose assay, as well as quenching the stable free radical DPPH and the ABTS radical cation. Additionally the *P. palmata* extract inhibited lipid oxidation, in a dose dependent manner, in a linoleic acid emulsion system (Yuan et al., 2005).

1.10.2.1 Pigment bioactivity

Phycobiliproteins are easily isolated and have significant antioxidant, anti-inflammatory, hepatoprotective and free radical scavenging properties (Khattar & Kaur, 2009; Yabuta et al., 2010). Phycoerythrin has been reported to have hepatoprotective and antioxidant activities in rats (Soni et al., 2009). The biological properties of phycocyanin includes antioxidant, anti-inflammatory, neuroprotective and hepatoprotective activities (Eriksen, 2008). When *in vitro* antioxidant activity was assessed, phycocyanin was able to scavenge alkoxyl, hydroxyl and peroxyl radicals and inhibited microsomal lipid oxidation induced by Fe^{+2}-ascorbic acid (Sekar & Chandramohan, 2008).

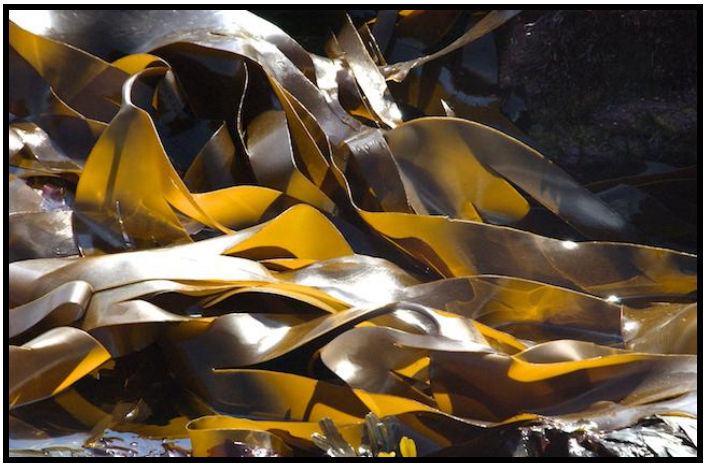
Carotenes are known to be reactive towards singlet oxygen and antioxidant activities β-carotene has been supported by a large body of research (Miller et al., 1996). The mechanisms of action of β-carotene include the ability to quench highly reactive singlet oxygen under certain conditions and also block free radical-mediated reactions (Bendich & Olson, 1989; Burton, 1989). Edge et al. (1997) has reviewed the reactivity of carotenoids with singlet oxygen and the interaction of carotenoids with a range of free radicals. In addition, many studies have confirmed a variety of health benefits including reducing the risk of human disorders (e.g. cancer) involving free radicals by preventing oxidative damage (Christaki et al., 2013).

1.10.2.2 Antimicrobial bioactivity

In general, many species of red seaweeds demonstrate moderate *in vitro* antimicrobial activity against a number of gram positive and negative bacteria, however limited antimicrobial data exists for whole or extracts of *P. palmata*. In most cases, *in vitro* antimicrobial activity has been established using simple assays such as disk diffusion or tube dilution method. Additionally, no scientific literature exists demonstrating antibacterial activity of seaweed extracts in food products (Gupta & Abu-Ghannam, 2011).

Dichloromethane, methanol and water extracts of *P. palmata* were ineffective at inhibiting microbial growth of five fish pathogenic bacteria strains. In the same study, extracts of other species of red seaweeds including: *Asparagopsis armata*, *Ceramium rubrum*, *Drachiella minuta*, *Falkenbergia rufolanosa*, *Gracilaria cornea* and *Halopitys incurvus* were most effective at inhibiting bacterial growth (agar diffusion assay), presumably due at least in part to lipophilic halogenated compounds extracted from the seaweeds (Bansemir et al., 2006). In another study, methanol, acetone and ethanol extracts of *P. palmata* inhibited the growth of *Listeria monocytogenes*, *Salmonella abony*, *Enterococcus faecalis* and *Pseudomonas aeruginosa*. The effectiveness of the extracts on certain bacteria was greatly influenced by the solvent of extraction. Methanolic extracts had double the antimicrobial activity against *L. monocytogenes* than acetone and ethanol extracts, while the ethanol extract was most effective at inhibiting the growth of *P. aeruginosa* (Cox et al., 2010).

1.11 Brown seaweed (*Laminaria digitata*) - Occurrence, habitat and structure



Laminaria digitata
[a]Guiry & Guiry (2014).

Kelps are the largest seaweeds and belong to the order Laminariales. *Laminaria digitata* is commonly found in low waters around Ireland, North Europe and Eastern North America (Morrissey et al., 2001). Kombu is the most common name for *L. digitata* species, however Kombu is often used as the common name for other kelps such as *Saccharina japonica* (MacArtain et al., 2007). In clear water, *L. digitata* has been found at depths up to 10 m. *L. digitata* thrives in fairly exposed areas as well as more sheltered areas with strong tidal currents, although it can be sensitive to low levels of salinities. Generally, *L. digitata* is found in waters with a salinity level above 20% (Morrissey et al., 2001). Golden brown in colour, *L. digitata* grows up to 2.5 m long and 60 cm wide. The frond of *L. digita* is flat, large, rubbery and smooth with finger-like sections and a holdfast which is a cluster of thin, branching, root-like processes (Guiry & Guiry, 2014). The cell wall of *L. digitata* is composed of cellulose, alginate and sulphated fucans. For the purposes of this review the structure of laminarin and fucoidan, reserve polysaccharides of *L. digitata*, will be discussed further.

1.11.1 Laminarin structure

Laminarin, a relatively low molecular weight storage polysaccharide (5-20 kDa), is composed of β-(1,3)-linked glucose, containing large amounts of sugars and a low fraction of uronic acids (Devillé et al., 2004). Higher quantities of laminarin are found in brown seaweeds during the winter months (Mišurcová et al., 2012). Laminarin contains two types of polymeric chains, i.e, the G-chains where glucose is attached to the end of the chain and the M-chains with mannitol (a sugar alcohol derived from the six carbon sugar D-mannose) as the terminal reducing end. Structural features of laminarin vary between seaweed species, including the degree of branching and degree of polymerization as well as the ratio of (1,3)- and (1,6)-glycosidic bonds (Anastasakis et al., 2011). The solubility of laminarin is influenced by the degree of branching, i.e. highly branched laminarin is soluble in cold water whereas lower levels of branching is only soluble in warm water (Rioux et al., 2007). While laminarin is soluble in water it does not gel or form any viscous solution (Kraan, 2012). Laminarin is resistant to hydrolysis in the upper gastrointestinal tract (GIT) due to forming complex structures stabilised by inter-chain hydrogen bonds and therefore is considered a dietary fibre (O'Sullivan et al., 2010). Subsequently laminarin is reported to be used by the endogenous intestinal microflora (Devillé et al., 2004; Gupta & Abu-Ghannam, 2011a).

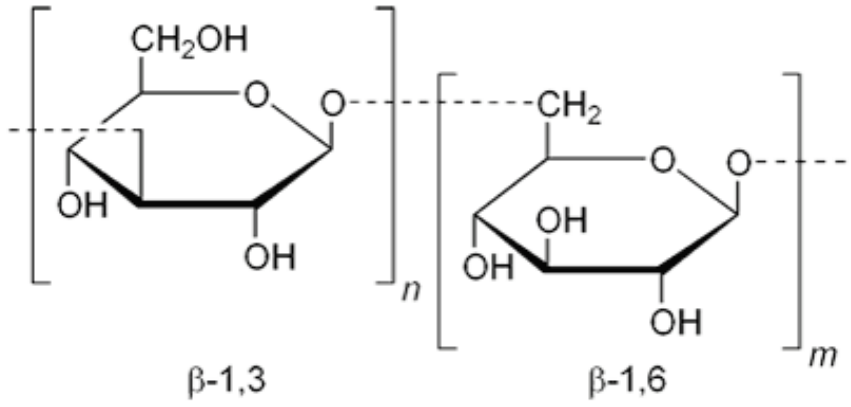


Figure 1.3. Laminarin structure from *L. digitata*.
Source: O'Sullivan et al. (2010).

1.11.2 Fucoidan structure

Fucoidan was first isolated in 1913 and possesses a diverse range of biological activities (Ale et al., 2012). Despite the vast amount of research on fucoidan, formally "fucoidin", no consistent basic structure of fucoidan exists (Ale & Meyer, 2013; Li et al., 2008). The structure of fucoidan is considered extremely complex and heterogeneous in composition in comparison to related polymers found in marine invertebrates (Balboa et al., 2013). The structure has been described to vary significantly between seaweed species and even within the same species (Anastasakis et al., 2011). Determination of the exact chemical structure of seaweed polysaccharides is often a complex task often due to the sulphated constituents (Usov & Zelinsky, 2013).

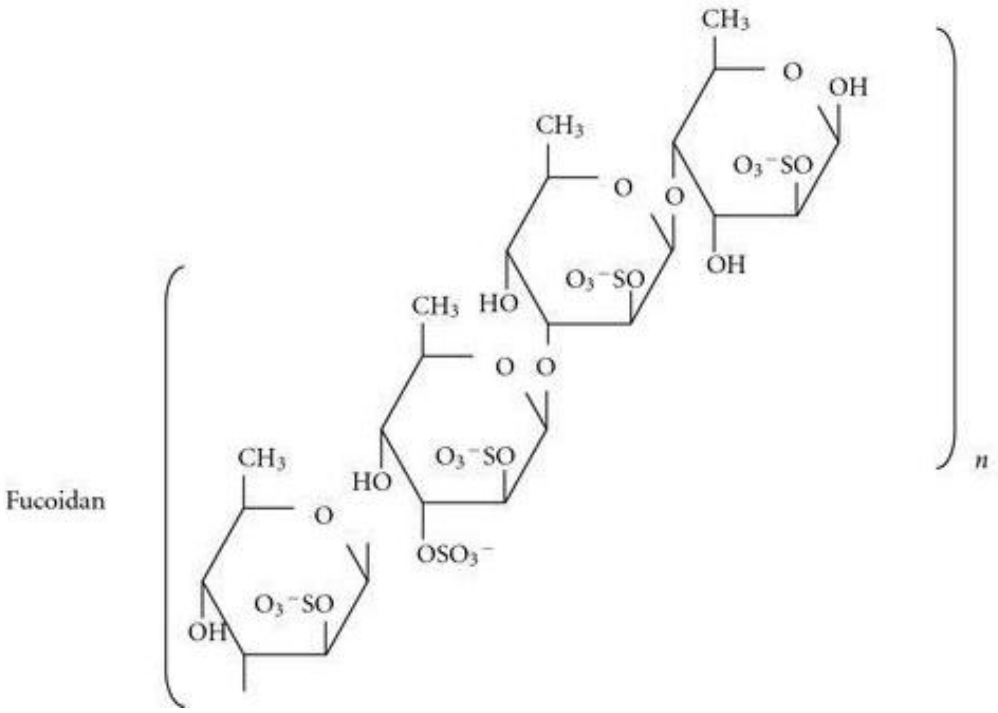


Figure 1.4. Fucoidan structure from *L. digitata*.
Source: Cumashi et al. (2007).

Some studies have shown a correlation between the depth at which brown seaweed grow and the content of fucoidan (Berteau & Mulloy, 2003). Various structural characteristics, such as linkage types, of fucoidan depend on species thus making it

difficult to define with precision (Kim, 2012). The backbone of fucoidan extracted from the brown seaweed species of the order Laminariales have been reported to mainly consist of α(1, 3)-linked L-fucopyranose residues with sulphates at the C-2 position (Anastyuk et al., 2012). The ratio of fucose to sulphate esters and the number of monosaccharides varies between species (Li et al., 2008). In addition to fucose and sulphates, fucoidan extracted from *Laminaria digitata* is reported to contain xylose, mannose, glucose, galactose and uronic acid in minor amounts (Li et al., 2008). A summary of the chemical composition of fucoidan extracted from *L. digitata* is presented in Table 1.12 (Cumashi et al., 2007). The exact molecular mass of fucoidan is unknown as it depends on seaweed species, extraction method and environmental conditions, but an average of 20,000 Da has been reported in the literature (Li et al., 2008).

Table 1.12. Composition of fucoidan extracted from *Laminaria digitata* (% w/w)[a].

Fucose	Xylose	Mannose	Glucose	Galactose	Uronic acids
30.1	1.9	1.7	1.4	6.3	7.0

[a]Cumashi et al. (2007).

Fucoidan is soluble in water and in acid solution (Rioux et al., 2007; Rupérez et al., 2002). Extraction methods for fucoidan can be as simple as hot water or the more commonly applied acid extraction or a combined hot acidic extraction with ethanol precipitation (Ale & Meyer, 2013; Li et al., 2008). Structural and compositional traits and subsequently the biological activities are significantly influenced by extraction parameters (Ale & Meyer, 2013). Extraction methods for fucoidan from brown seaweeds and the relationship between extraction and structure have been reviewed previously by Ale et al. (2011). Hahn et al. (2012) reviews other novel procedures for the extraction of fucoidan.

1.11.3 Composition of *Laminaria digitata*

A compositional analysis of *L. digitata* is presented in Table 1.13. *Laminaria spp.* contain high levels of carotenoids and vitamin E (Jaspars & Folmer, 2013). The characteristic brown colour of *L. digitata* is due to fucoxanthin, one of the most abundant carotenoids in nature (Chandini et al., 2008). Like all kelps, *L. digitata* is rich in iodine and an excellent source of calcium and magnesium (Morrissey et al., 2001).

Table 1.13. Compositional analysis of *Laminaria digitata*.

Macro/micronutrient	**%**
Water[x]	73-94%[c]
Protein[y]	8-14%[a]
Lipid[y]	1%[b]
Carbohydrate[y]	48%[d]
Ash[y]	21-35%[c]
Laminarin	0-18%[c]
Mannitol	4-16%[c]
Vitamin C	12-18 ppm[b]
Vitamin B1	5 ppm[b]
Vitamin B2	22 ppm[b]
Vitamin B3	34 ppm[b]
Vitamin B12	0.6-0.12 ppm[b]
Calcium	12,400-13,200 ppm[b]
Iodine	800-5,000 ppm[b]
Iron	50-70 ppm[b]
Magnesium	6,400-7,860 ppm[b]
Manganese	1-16 ppm[b]
Sodium	2-5.2%[b]

[x]wet weight basis.
[y]dry weight basis.
[a]Burtin (2003).
[b]Morrissey et al. (2001).
[c]Guiry & Guiry (2014).
[d]Rhatigan (2009).

1.11.4 Bioactive compounds present in *Laminaria digitata*

In general, several studies have demonstrated that brown seaweed has higher antioxidant capacity than green and red seaweeds (Jiménez-Escrig et al., 2011; Prabhasankar et al., 2009). Brown seaweeds are a source of many antioxidant

compounds such as phlorotannins (phloroglucinol, eckol), ascorbic acid, catechins (catechin, epigallocatechin, epigallocatechin gallate), tocopherols (α-, γ-, δ-tocopherols), laminarin, fucoidan and carotenoids (α- and β- carotene, fucoxanthin) (Holdt & Kraan, 2011; Zubia et al., 2009). The biological activity of brown seaweeds and their components has been extensively reviewed by Gupta & Abu-Ghannam (2011a). A dose-dependent *in vitro* antioxidant activity using a wide variety of assay (DPPH, FRAP, ABTS, reducing power, etc.) has been observed for crude extracts as well as polysaccharide fractions from brown seaweeds. Extracts show a typical behaviour which reaches a plateau at a given concentration; however phenolic compounds reach maximum protection at significantly lower levels than polysaccharide compounds (Balboa et al., 2013).

1.11.4.1 Polyphenolic bioactivity

Polyphenols or phenolic compounds of brown seaweeds include: phlorotannins (phloroglucinol, eckol), catechins (catechin, epigallocatechin, epigallocatechin gallate), tocopherols (α-, γ-, δ-tocopherols), ascorbic acid and carotenoids (α- and β- carotene, fucoxanthin) and are characterized structurally by the presence of several hydroxyl groups on aromatic rings (Tierney et al., 2010). Polyphenols are secondary metabolites which protect cell constituents of seaweeds against oxidative damage. Seaweed polyphenols, also called phlorotannins, differ from terrestrial plant polyphenols as they are derived from phloroglucinol units (1,3,5-trihydroxybenzine) (Jaspars & Folmer, 2013). Phlorotannins only occur in brown seaweeds with levels ranging from 5-15% DM. Balboa et al. (2013) has reviewed the antioxidant properties of phenolic compounds from crude extracts isolated from brown seaweeds. Seaweed phlorotannins are associated

with a wide range of biological activity, especially antioxidant activity, and constitute an extremely heterogeneous group of molecules (Burtin, 2003).

1.11.4.2 Laminarin bioactivity

Laminarin and some derivatives are attracting interest due to potential biological activities. In general, the biological activities of laminarin are considered dependant on the molecular structure, including molar mass, degrees of polymerization, branching and length of branch (Choi et al., 2011). Modification of the chemical structure of laminarin can result in enhanced biological activities, for example partial depolymerisation of laminarin sometimes leads to increased bioactivity (Usov & Zelinsky, 2013). Devillé et al. (2004) studied the fate of laminarin after digestion and the potential role of laminarin as a dietary fibre. Like other β-glucans, laminarin can serve as a prebiotic and modulate immune function (Devillé et al., 2007). In addition, laminarin has also been reported to possess antitumor, antimicrobial, anticoagulant and antioxidant activities (Gupta & Abu-Ghannam, 2011a). Choi et al. (2011) reported that low molecular weight laminarin has enhanced biological activities including enhanced antioxidant activity. Although antioxidant potential is reported for laminarin, Chattopadhyay et al. (2010) demonstrated that it is less potent than other antioxidant components present in brown seaweeds.

1.11.4.3 Fucoidan (general) bioactivity

Seaweeds are among the richest natural, non-animal sources of known and novel bioactive sulphated polysaccharides. Low molecular weight fractions have been reported as more biologically active than crude extracts however no consensus has been reached regarding optimal molecular size (Anastyuk et al., 2012; Usov & Zelinsky, 2013). Fucoidan and fucose rich sulphated polysaccharides exhibit a range of biological

activities including immunomodulatory, antitumor, anti-inflammatory, antiviral, antithrombotic and anticoagulant effects (Ale et al., 2012; Li et al., 2008). The most significant bioactivities (anti-tumor, immunomodulatory, anti-coagulant, antithrombotic) and their relationship to fucoidan structures has been reviewed previously (Ale et al., 2011; Wijesinghe & Jeon, 2012). A multitude of reviews for the biological activities of sulphate rich polysaccharides and their impact on health and disease also exist (Bhakuni & Rawat, 2005; Blunt et al., 2009; El Gamal, 2010; Fernandes et al., 2014; Folmer et al., 2010; Mayer & Lehmann, 2001; Mohamed et al., 2012; Rindi et al., 2012; Venugopal, 2008).

1.11.4.4 Fucoidan (antioxidant) bioactivity

Recently, the antioxidant activity of chemically anionic sulphated polysaccharides, has gained considerable attention. Fucoidan demonstrated higher antioxidant potential than sulphated galactans such as carrageenan (de Souza et al., 2007). Various *in vitro* tests (DPPH, FRAP, NO, ABTS radical scavenging, lipid peroxide inhibition, superoxide radical and hydroxyl radical scavenging) have confirmed the antioxidant activity of fucoidan from a variety of brown seaweed species (Costa et al., 2010; Kim et al., 2014; Rupérez et al., 2002; Wang et al., 2008; Wang et al., 2010a; Zhao et al., 2008). Furthermore, sulphated polysaccharides from seaweeds are considered to be important free-radical scavengers and antioxidants for the prevention of oxidative damage (Ngo et al., 2011). Much of the work to-date has been carried out on crude fucoidan preparations with a lack of characterisation of the extracts tested and limited information on the extraction procedures used, making the relationship between activity and composition difficult to ascertain (Li et al., 2008).

A relationship between the structure and antioxidant activities of sulphated seaweed polysaccharides is not clearly established; but the importance of monosaccharide composition, sulphate content, position of sulphate ester groups and molecular size has been reported previously (Chattopadhyay et al., 2010; Costa et al., 2010; Li et al., 2008). Sulphates linked to monosaccharides will influence biological activity as well as the functionality of the polysaccharide (Rioux et al., 2010). Lower molecular weight and high sulphate content has been linked to stronger radical scavenging activities and reducing power than high molecular weight sulphated polysaccharides (Patel, 2012; Qu et al., 2014; Tierney et al., 2010). Acid-soluble fractions containing fucans showed the highest antioxidant activities compared to those containing alginate and laminarin in *Turbinaria conoides* and *Fucus vesiculosus* seaweeds. Additionally, the fraction containing the highest level of sulphates was significantly correlated to antioxidant activity either by reducing power or radical scavenging assay (Jiménez-Escrig et al., 2014).

Conversely, some research speculates that the antioxidant activity of fucoidan is not correlated to the content of substituent groups but rather to the impurities such as polyuronic acids, proteins and phenolic compounds present in seaweed extracts (Imbs et al., 2014; Mak et al., 2013). Many studies indicate acidic extraction of seaweed produces a complex mixture of glucans, fucoidan and alginic acid (Zvyagintseva et al., 2005). Polyphenols often co-extracted with fucoidan are difficult to fully eliminate during purification processes due to covalent irreversible bonds. Dragar & Fitton (2006) stated polyphenols may enhance fucoidan bioactivity. Phenolic fractions achieve maximum protection of oxidisable substrates at lower levels than polysaccharide components from brown seaweeds.

Chemical modifications of fucoidan have resulted in higher antioxidant activity. Over sulphated, acetylated, benzoylated, phosphorylated and aminated derivatives of fucoidan exhibited higher antioxidant activities than fucoidan in a number of *in vitro* tests (superoxide, hydroxyl and DPPH free radical scavenging activity and reducing power). The differences in mechanism of action was linked to the different substituted groups (Wang et al., 2009a). Phosphate and amino groups influenced the mechanism of the antioxidant activity of fucoidan derivatives (Wang et al., 2009b).

A small number of *in vivo* studies demonstrate antioxidant activity of fucoidan. Fucoidan from *L. japonica* prevented an increase in lipid oxidation levels in serum, liver and spleen tissues of diabetic mice (Vo & Kim, 2013). Fucoidan protected against cellular oxidative stress, in a concentration dependant manner, by inhibiting lipid oxidation of renal epithelial cells (So et al., 2014). Similarly, fucoidan from *L. japonica* reduced oxidative stress in rats with adenine-induced chronic kidney disease by activation of antioxidant defences (Wang et al., 2008). The increase in antioxidant status of both serum and cecum were reported in healthy rats fed a diet of *Saccharina latissima* due to the sulphated polysaccharides associated with the seaweed (Jiménez-Escrig et al., 2012). Additionally, crude extracts from *L. digitata* were effective at inhibiting lipid oxidation of sunflower oil (Le Tutour et al., 1998).

1.11.4.5 Antimicrobial bioactivity

Dubber and Harder (2008) investigated antibacterial effects of hexane and methanol extracts of the macroalgae *L. digitata* on twelve marine and seven prominent fish pathogenic bacteria using a highly sensitive growth inhibition assay. Hexane extracts of *L. digitata* were effective at inhibiting growth of 10 out of 12 marine and 6 out of 7 fish pathogenic bacteria. The antimicrobial properties of several Irish edible brown

seaweeds using a microtitre method was determined and methanol extracts of *L. digitata* showed good antibacterial activities against *Listeria monocytogenes, Salmonella abony, Enterococcus faecalis* and *Pseudomonas aeruginosa* (Cox et al., 2010; Gupta et al., 2010). In another study, ethanol and acetone extracts of Icelandic *L. digitata* demonstrated low to moderate antibacterial activity against *Staphylococcus aureus, L. monocytogenes, Escherichia coli, P. aeruginosa* and *Candida albicans* (Qiao et al., 2010).

Antibacterial activities of brown seaweed extracts containing polysaccharides have been extensively studied by several researchers (Rajasulochana et al., 2009; Salvador et al., 2007; Seenivasan et al., 2010). Antimicrobial activity of brown seaweed polysaccharides has been linked to the chemical structure and sulphate content. However several species of brown seaweeds with high sulphate content showed differences in antimicrobial activity (Berteau & Mulloy, 2003; Chandrasekaran et al., 2014; Chauhan & Kasture, 2014; Mohsen et al., 2007). In general, brown seaweed derived polysaccharides markedly inhibit *Staphylococcus*, *Bacillus*, *Proteus* and *E. coli*. In particular, *P. aeruginosa* and *Fusiform bacillus* are most sensitive to brown seaweed polysaccharides (Lee et al., 2013; Li et al., 2010). Limited antimicrobial studies exist on crude extracts containing fucoidan. A crude fucoidan extracted from *Sargassum polycystum* showed antibacterial activity against *Vibrio harveyi*, *S. aureus* and *E. coli* at concentrations of 12, 12 and 6 mg/ml, respectively (Chotigeat et al., 2004). Crude fucoidan was evaluated for its antimicrobial activities against 11 common bacterial species present in the oral cavity. Fucoidan exhibited high antimicrobial activity against all tested bacteria with the strongest antimicrobial activity against anaerobic bacteria, *P. gingivalis* (Lee et al., 2013).

SECTION 3: SEAWEEDS AS FUNCTIONAL INGREDIENTS

1.12 Functional foods

The demand and popularity for functional foods, because of their associated health benefits, continue to grow in popularity yearly. Functional foods, also known as nutraceuticals, are defined in a number of different ways by legislative bodies. Goldberg (1994) outlined three primary criteria which a food must satisfy in order to achieve functional status, it must be: a food (not capsule, powder or tablet), consumed as a part of a daily diet, and once ingested, regulate specific processes such as enhancing biological defence mechanisms, preventing and treating specific disease, controlling physical and mental conditions, and delaying the ageing process.

Functional foods were first developed in Japan in the 1980s. In the late 1990s, Functional Foods Science in Europe (FUFOSE) was set up by a working group coordinated by the European Section of the International Life Science Institute, with European Commission support (Tierney et al., 2010). The action of functional foods is based on the use of functional ingredients. Health benefits of different food components have been recognised for some time now, but it is only more recently that their role in the treatment and prevention of various diseases, or impact on the ageing process has been established (Jiménez-Colmenero et al., 2001). The functional components of different foods and their impact on health and disease is well reviewed by Abuajah et al. (2014).

To date, the development of functional food products has been primarily associated with the dairy industry. This is not particularly surprising given that milk is composed of many multifunctional components which have physiological functions beyond just nutrition. Functional dairy products are some of the most recognised functional foods on supermarket shelves today, including: a wide variety of yogurts and spreads containing added bacterial cultures, plant sterols and stanols etc. Additionally,

the beverages market has been a fast growing sector with many non-alcoholic functional drinks containing vitamins or other functional ingredients now in production (Hafting et al., 2012).

1.12.1 Seaweed as functional antioxidant ingredient

Some of the most important and frequently used functional ingredients used in foods include: probiotics, prebiotics, plant antioxidants and vitamins (Grajek et al., 2005). In the past number of years, the functional properties of many plant extracts have been investigated for their potential use as novel nutraceuticals as concerns increase regarding the safety and toxicity of synthetic antioxidants (O'Grady et al., 2008). Natural antioxidants extracted from plants such as rosemary, sage, tea, soybean, citrus peel, sesame seed, olives, carob pod, and grapes can be used as alternatives to the synthetic antioxidants because of their equivalent or greater effect on the inhibition of lipid oxidation (Tang et al., 2001). The Japanese Ministry for Health and Welfare has identified twelve broad groups of ingredients that they consider as having potential beneficial effects on human health: dietary fibre; oligosaccharides; sugar alcohols; amino acids, peptide and proteins; glycosides; alcohols; isoprenoids and vitamins; cholines; lactic acid bacteria; minerals; polyunsaturated fatty acids; and others. Antioxidants fall into the last category which includes all others that are not listed in the first eleven (Goldberg, 1994).

Interest in the use of seaweed as functional foods, especially in relation to the range of antioxidant components is highlighted by the publication of several patent applications as well as many literature studies (Chapman & Chapman, 1980; Nisizawa et al., 1987; Shahidi, 2009). These include: a fucoidan antioxidant beverage, fucoidan containing antioxidant capsules and seaweed in food products like pasta and sausages

(López-López et al., 2009a, 2009b; Prabhasankar et al., 2009; Tierney et al., 2010). The advantages of using seaweeds as a source of natural ingredients for functional foods include: ease of cultivation, rapid growth rate and the potential to control production of bioactive compounds through manipulating cultivation conditions (Plaza et al., 2008).

1.13 Muscle foods as vehicles/carriers of functional ingredients

As the acceptance of the functional food market grows, it permits food suppliers to consider other foodstuffs as potential sources for functional foods. Primarily among these is muscle based foods (meat, poultry and fish) which are as popular, if not more popular, than dairy products in certain regions of the world, are no less rich in multifunctional components than dairy products and equally function to aid in the delivery of added nutraceuticals to consumers. Some 36-40% of total calories in industrialised countries diets come from fat while nearly half of that is from meat intake (Byers et al., 1993; Sheard et al., 1998). Like dairy products, meat contains vital nutrients for human health but unlike many other foods, meat is much less allergenic to consumers. Taking this into consideration, it makes sense to focus on the meat industry as a way to incorporate healthier foods into consumer's diets. Such products could potentially open up a new market for the meat industry. The use of functional ingredients in meat and meat products has been well reviewed (Olmedilla-Alonso et al., 2013; Zhang et al., 2010). A summary of current strategies to produce functional meat products is presented in Table 1.14.

Table 1.14. Production of functional meat products[a].

Production of functional meat products through dietary supplementation	Addition of functional ingredients during processing	Production of functional components during processing
Conjugated linoleic acid	Vegetable proteins (soy, whey and wheat)	Curing (production of peptides and free amino acids)
Vitamin E	Fibre	Fermentation (production of antibacterial compounds and probiotics)
Omega-3 fatty acids	Herbs and spices (rosemary, green tea, clove, garlic, sage and oregano)	Enzyme hydrolysis of proteins (production of peptides)
Selenium	Probiotics and lactic acid bacteria	

[a]Zhang et al. (2010).

1.13.1 Fish as carrier of functional ingredients

Fisheries remain an important source of food, employment and revenue in many countries and communities (FAO, 2013b). The image of fish to consumers is positive as it provides a healthy intake of quality protein, fat, vitamins and minerals. International and national guidelines recommend at least two fatty fish dishes per week as part of a healthy diet. Health benefits attributed to fish intake include improvement in cardiovascular health and arthritis symptoms, lower blood pressure and reduced risk of cancer (Kinsella, 1986). As a result, fish and fish products are increasingly promoted as functional foods. Additionally, fish and their by-products are an excellent source of bioactive compounds which are extracted and added to a range of other foods to enhance functionality in terms of human health (Gormley, 2013). While inherently functional, fish are being considered as a suitable carrier of additional functional ingredients to further enhance health benefits for consumers and protect/enhance the stability of beneficial lipids (Gormley, 2006). One aspect of improving the nutritional profile of fish

is by enriching fish products with dietary fibres, antioxidant dietary fibres or prebiotics (Diaz-Rubio et al., 2011; Sánchez-Alonso et al., 2008).

1.13.2 Pork meat as carrier of functional ingredients

Due to consumer demand for healthier meat products, the meat industry has steadily worked to reduce the fat content of meat, especially in beef and pork products (Bryhni et al., 2002). Improved breeding and husbandry practices have resulted in 30% less fat present in retail cuts of pork than during the 1970s. The US pork industry has capitalised on this by marketing pork as a light and nutritious alternative to chicken. As a result of marketing, the yearly consumption of pork in the US has remained stable at about 47.8 pounds per person per year since 1970 (Resurreccion, 2004). Research, to date, indicates that consumers are now less likely to perceive pork negatively in terms of fat, calories and cholesterol. Even though pork meat is high in valuable and essential nutrients such as protein, B vitamins, iron, zinc and oleic acid, some doubt remains about the nutritional benefits of pork compared to chicken (Verbeke et al., 1999). The perception of "healthiness", health benefits and risks are the most commonly acknowledged reasons for reducing meat consumption (Grunert et al., 2004). With consumer's perception of "healthiness", there are plenty of opportunities to add functional ingredients to further enhance the health aspects of pork meat. One aspect the meat industry has already focused on is through manipulating the fatty acid profile (increasing PUFA content at the expense of saturated fatty acids (SFA)) with dietary vegetable oils. Another area to enhance the perception of pork is through manipulation of diet composition in order to improve the quality and eating characteristics such as leanness, taste, odour, tenderness and juiciness (Verbeke et al., 1999).

1.14 Seaweed processing and manufacture of functional ingredients

Traditionally, seaweeds have been subjected to some form of processing due to their compositional and textural properties. Seaweeds are sundried over a net or a tarpaulin on the ground, in order to render them palatable for human consumption and reduce water activity to minimise deterioration from chemical reactions and microbial activities before being used as food. Before consumption, seaweeds can be rehydrated to restore a palatable texture. Owing to the high level of unsaturated fatty acids, seaweeds deteriorate with in a few days of harvesting if they are not properly preserved (Paull & Chen, 2008).

Long drying times and high temperatures have been linked to undesirable thermal degradation in vegetable products. Similar effects would be anticipated in the drying of seaweeds. Likewise, losses in bioactivity could be expected if time and temperature parameters are not adequately controlled and optimised (Abu-Ghannam & Cox, 2014). Processing of food and food ingredients, especially thermal treatment, often exerts a major effect on their constituents, including biological activity (Shahidi, 2009). Processing of various plant bioactives has demonstrated both positive and negative effects on the level of bioactivity. Heat treatment of lycopene, the main carotenoid of tomatoes, enhances the bioavailability (Rao & Ali, 2007). Similarly, roasting canola/rapeseed or sesame seeds enhances the stability of PUFAs due to the formation of Maillard reaction products (Jeong et al., 2004; Shahidi et al., 1997). On the other hand, processing of soybeans to produce protein concentrates and isolates has resulted in a substantial decrease in isoflavone content (Jackson et al., 2002). High temperature cooking and drying has been reported to cause a significant loss of vitamin C in brown seaweeds (Venugopal, 2011). Conversely, Yoshiki et al. (2009) reported heat treatment over 100°C

enhanced DPPH activity of low molecular weight fractions of a water soluble *Porphyra yezoensis* extract.

In a study which investigated optimal storage conditions of freshly harvested seaweed, Paull & Chen (2008) reported that red seaweed (*Gracilaria spp.*) submerged in seawater at 15 to 17°C in the dark extended postharvest life from 4 days to approximately 30 days. Alternatively, treating *G. tikvahiae* and *G. parvispora* with hot seawater at 38-42°C for 5 min was beneficial in extending postharvest life from about 4 days to 5 and 8 days, respectively. Other treatments such as postharvest dip in artificial seawater supplemented with 1 or 10mM calcium, potassium, sodium, ammonium as the nitrate salt were ineffective at extending postharvest life. Paull & Chen (2008) concluded that light and water temperature were two major factors in extending the shelf-life of seaweed post harvest.

To date, little information on the effects of drying or heating on nutritional composition or biological activity has been reported for seaweeds. In a recent study, drying *H. elongata* for 24 hours, at four temperatures (25, 30, 35 and 40°C), resulted in a reduction in total phenolic content, regardless of the temperature used. A higher loss of phenolic compounds was observed at lower temperatures, which was attributed to the changes in seaweed composition and content. Dehydration also resulted in a loss of antioxidant activity. DPPH radical scavenging activity was significantly lower in *H. elongata* dried at 25°C compared to 40°C (Abu-Ghannam & Cox, 2014).

Limited studies suggest air drying can prevent seaweeds from further loss of bioactivity before additional processing. After 40 minutes of thermal processing at 80°C or 100°C of fresh *H. elongata*, the total phenolic content was reduced by up to 85% (Cox et al., 2012; Cox et al., 2011). However if the seaweed was subjected to a drying step prior to hydrothermal processing, losses were minimized. Cox et al. (2012) reported

drying at 25°C for 12 hours followed by hydrothermal processing at 100°C reduced the required cooking time to achieve palatability to 25 minutes and only 9% of the total phenolic content was lost as compared to fresh seaweeds. Similarly, semi-dried seaweeds (at 40°C for 2 hours) resulted in increased phytochemical content and enhanced DPPH free radical scavenging activity, compared to fresh seaweeds (Gupta et al., 2011b).

1.14.1 Seaweed inclusion in fish pellets

Compound fish feeds are formulated according to precise nutrient specifications to ensure product quality and nutrient balance for maximum growth effects, as well as easy and cost effective application (FAO, 2014). Moist and dry pelleted feeds exist, although the use of dry pellets is more common as the product is more practical for feed manufacture's due to shelf-life considerations. A number of dry fish feeds based on fish meal are available commercially (Das et al., 1993). Fish meal is often partially replaced with other ingredients (fish waste, vegetable material etc) in order to reduced feed costs (Soler-Vila et al., 2009). During the last decade, the use of extruded pellet diets for fish has markedly increased due to the pellets having superior water stability, floating properties and higher energy yields (Ammar et al., 2008). A schematic reproduction of the manufacture process of dry fish pellets containing added seaweed used in Chapters 2 and 3 is presented in Figure 1.5. Seaweed is first washed and dried before being added to the fish feed formulation which includes fish meal, fish oil, binding agents, vitamins and minerals. Grinding of the mix is carried out before water is added to form a paste which is then forced through an extruder and cut to form pellets of the required length. Finally the pellets are oven dried (Das et al., 1993).

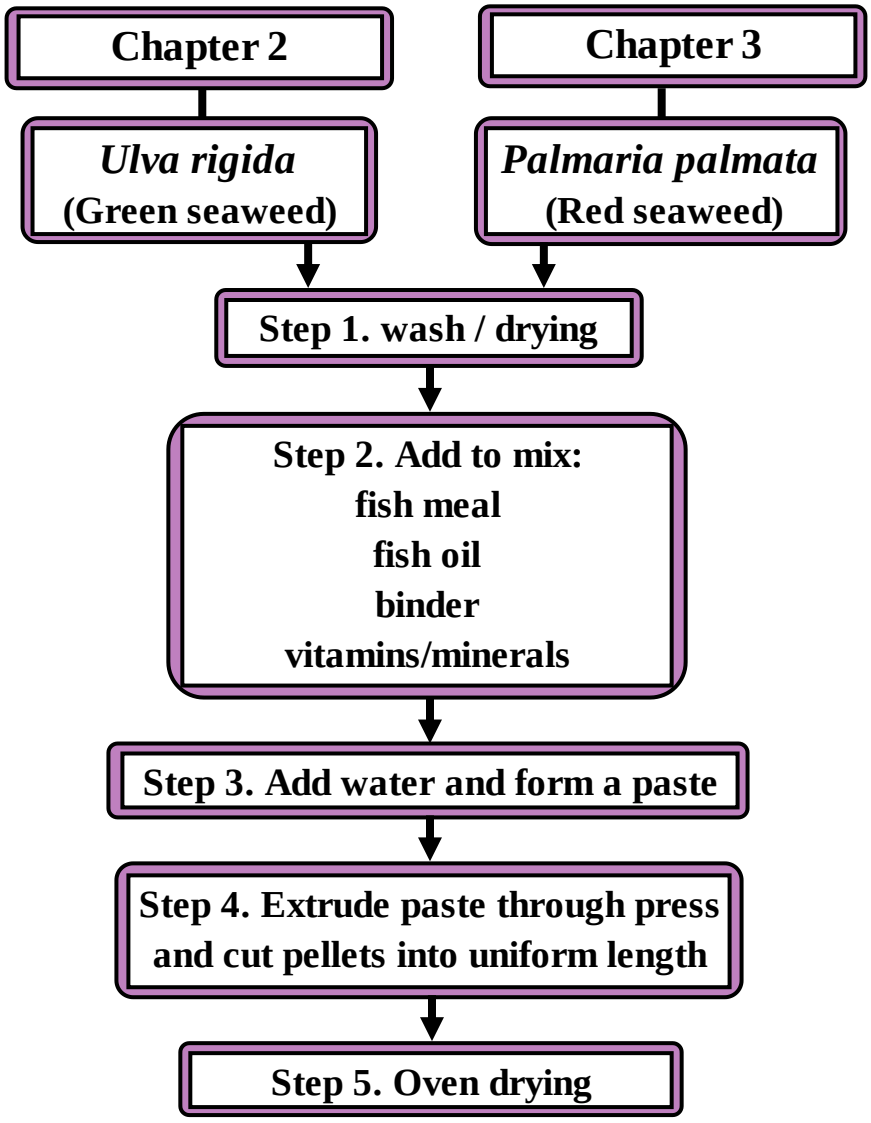


Figure 1.5. Manufacture of dry fish feed pellets containing seaweed.

1.14.2 Techniques used for the manufacture of seaweed extracts

Classical techniques have been traditionally employed to extract compounds with bioactivity from seaweed including: Soxhlet, liquid–liquid extraction (LLE), solid–liquid extraction (SLE) and other techniques based on the use of organic solvents. Although such techniques are well established and routinely used, a number of draw backs exist in relation to time, labour, automation, high volumes of solvents, etc. Such factors influence reproducibility, selectivity and provide low extraction yields (Herrero et al., 2013). These

shortcomings can be overcome by the use of new more advanced extractions techniques. The most important advanced extraction techniques include supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), ultrasound/sonication extraction (UAE), microwave-assisted extraction (MAE), accelerated solvent extraction (ASE®), water extraction and particle formation on-line (WEPO®), enzyme assisted extraction (EAE). These new techniques are faster, more selective and environmentally friendly as the use of toxic solvents is highly limited (Duan et al., 2006; Duarte et al., 2014).

The extraction technique utilised depends on the solubility of the desired compound (López et al., 2011). Furthermore, food grade solvents and enzymes are required for the preparation of antioxidant extracts which will be used as ingredients in food products (Duarte et al., 2014). SFE is an ideal extraction method for food grade extracts of non-polar compounds, however it is associated with high costs and low extraction yields (Herrero et al., 2013). Examples of different extraction techniques for different compounds from various seaweeds are presented in Table 1.15.

1.14.2.1 Manufacture of polysaccharide rich extracts containing laminarin and fucoidan

A variety of solvents and extraction methods are employed to extract water soluble polysaccharides (laminarin and fucoidan) from seaweeds. Wet- and spray-dried seaweed extracts containing laminarin and fucoidan used in the present study (Chapters 4-7) were manufactured by Bioatlantis (Tralee, Co. Kerry, Ireland). The extracts were prepared from brown seaweed (*Laminaria digitata*) using an acid extraction technique, details of which are industry confidential. A basic representation schematic of the extraction procedure and manufacture of the extracts is presented in Figure 1.6. Generally, polysaccharides are extracted from milled dried seaweeds by selective solvents

Table 1.15. Extraction techniques employed to isolate seaweed compounds.

Extraction technique	Seaweed	Compound isolated	Reference
Supercritical fluid extraction (SFE)	*Undaria pinnatifida*	fucoxanthin polyphenols	Roh et al. (2008).
SFE	*Scenedesmus obliquus*	chlorophyll	Choi et al. (1987).
Ultrasound/sonication extraction (UAE)	*Porphyra yezoensis*	phycobiliprotein	Zhu et al. (2008).
UAE	*Porphyra species*	Mycosporine-like amino acids (MAAs)	Tartarotti & Sommaruga (2002).
UAE	*Palmaria palmata*	MAAs	Yuan et al. (2009).
Microwave-assisted extraction (MAE)	*Fucus vesiculosus*	fucoidan	Rodriguez-Jasso et al. (2013).
Enzymatic and solvent extraction	*Sargassum filipendula*	fucoidan	Costa et al. (2011).
Solvent extraction, Silica column, Chromatography, Sephadex chromatography and HPLC	*S. siliquastrum*	Fucoxanthin	Heo & Jeon (2009)

such as ethanol, $CaCl_2$ and HCl to isolate desired compounds. Ethanol is frequently used to extract pigments and protein. $CaCl_2$ is then applied to precipitate alginates as well as extract laminarin and fucoidan. Fucoidan is often extracted by the use of HCl (Gamal-Eldeen et al., 2009; Ponce et al., 2003; Rioux et al., 2007; Zvyagintseva et al., 1999). The resulting wet-extract containing laminarin and fucoidan (L/F-WS) (Chapter 4-5) was spray-dried to form a spray-dried seaweed extract containing laminarin and fucoidan (L/F-SD) (Chapters 4, 6-7).

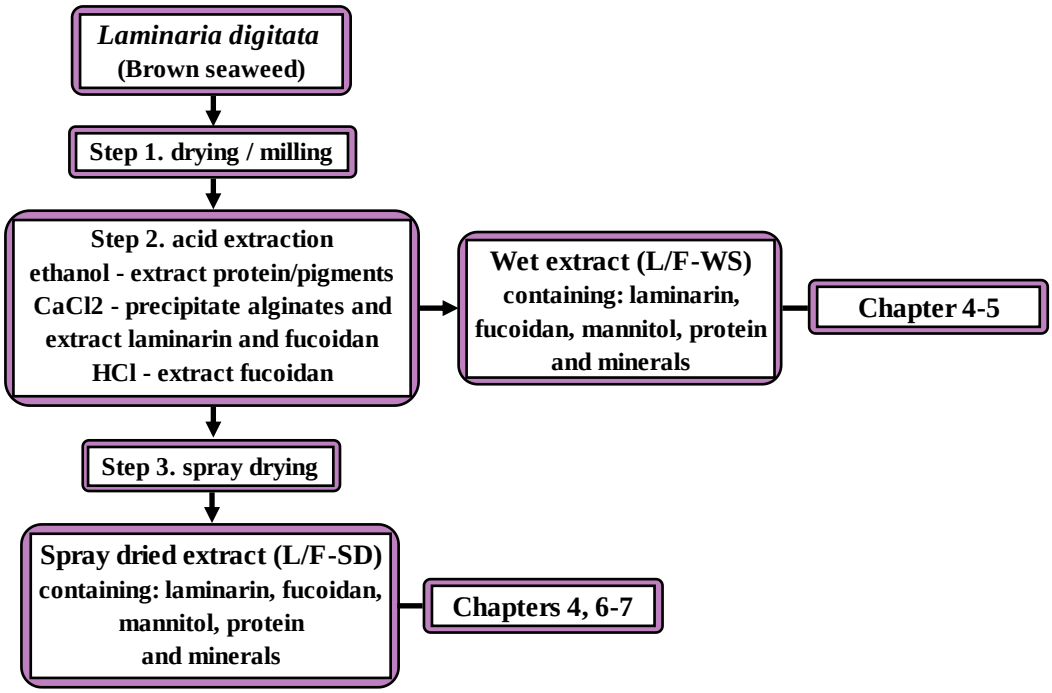


Figure 1.6. Extraction and manufacture schematic of a wet (WS) and spray-dried (SD) seaweed extract containing laminarin and fucoidan (L/F) isolated from *Laminaria digitata*.

1.15 Modes of incorporating seaweed / seaweed extracts as functional ingredients in muscle foods

Currently there are two main strategies used to manufacture meat products using functional ingredients: dietary supplementation or direct addition to meat during processing (Jiménez-Colmenero et al., 2001). The effect of functional ingredients added to animal diets may vary depending on the type of animal species and processing. Some functional ingredients are added to animal feeds in order to serve as a route to pass compounds into the circulatory system which are subsequently distributed and retained in muscle tissues (Toldrá & Reig, 2011). Dietary composition and feeding levels significantly impacts the ratio of fat to lean in pig and cattle carcasses (Hay & Preston, 1994). It is necessary to have the correct dietary fatty acid profiles for monogastric

animals such as pigs and poultry to produce a meat product with less saturated fat (Byers et al., 1993).

1.16 Supplementation of seaweed into animal diets

The use of seaweeds in animal feed comprised 1% of the global value of the seaweed industry in 2004 (Chojnacka et al., 2012). In Europe, the use of seaweeds in animal feed dates back to as early as Roman times. In Iceland, France and Norway, domestic animal diets were supplemented with seaweed to enhance nutritional value of the feed (FAO, 2003). The use of seaweeds as feed supplements has been reviewed by (Holdt & Kraan, 2011). Numerous experiments have demonstrated the nutritional benefits of seaweed supplementation in the diets of pigs, cows, sheep, chickens and other domestic animals, as well as in many aquatic organisms. Ruminant animals have been found to be the most suitable target for dietary seaweed inclusion as they are able to digest unprocessed seaweed cell walls (Kovač et al., 2013).

A number of *in vivo* studies exist with seaweeds added to the diets of farmed animals for nutritional purposes which focus on affects on growth performance and the health of the animal (van den Burg et al., 2013). The inclusion of seaweed extracts in animal feed has resulted in a variety of benefits including overall health improvement of feedstock, enhanced immune systems, favourable changes in gut microflora and increased milk production (Devillé et al., 2007; Leonard et al., 2011). In addition, many seaweed extracts have significantly enhanced the growth and immune systems of fish (Carter & Hauler, 2000; Chaiyapechara et al., 2003; Valente et al., 2006). Furthermore, the addition of antioxidant compounds to animal feed can protect against oxidative damage in living systems by scavenging oxygen radicals and also for increasing the stability of foods by preventing lipid oxidation in muscle post slaughter (Moure et al., 2001).

1.16.1 Supplementation of seaweed into fish diets

Fish aquaculture is a growth industry worldwide (Werner et al., 2004). As the demand for affordable fresh fish increases, so does the demand for fish feed. Fish meal (the main protein source) is an essential constituent of fish feed and is a substantial cost in fish farming, therefore producers are searching for alternative protein sources from plants (Holdt & Kraan, 2011). The use of seaweeds as an additive to fish diets and the effects on growth, performance and fish health has been reviewed (Hasan & Chakrabarti, 2009; Mustafa & Nakagawa, 1995).

For most fish species, 10-15% of the dietary protein can be met through the use of seaweeds without compromising the growth or feed efficacy of the fish. The use of seaweed as an additive to fish feed demonstrated positive results such as an increase in growth, improvement in physiological activity, improved stress response and disease resistance (Hasan & Chakrabarti, 2009). The use of *Porphyra*, *Ascophyllum* and *Ulva spp*. in fish meal exhibited the most pronounced effects on cultured sea bream growth performance. Results suggested that the use of seaweed as feed additive is an effective source of nutrients for fish (Holdt & Kraan, 2011; Mustafa & Nakagawa, 1995; Mustafa et al., 1995). The inclusion of ~3% or greater of *Macrocystis pyrifera* meal resulted in an increase of polyunsaturated fatty acids in the muscle in rainbow trout juveniles and subsequently a higher quality fillet (Dantagnan et al., 2009).

1.16.1.1 Supplementation of carotenoids into Atlantic salmon diets

After nutrition, an important aspect of salmon feed to consider is the addition of carotenoids. Carotenoids are responsible for the attractive red, yellow and orange colours of fish. Next to freshness, the pigmentation of Atlantic salmon (*Salmo salar*) flesh is regarded as the most important quality criterion (Decker et al., 2000). Fish cannot

synthesise carotenoids, therefore final flesh colour is influenced by dietary intake. The pigments primarily responsible for salmonids (trout, salmon and others) colour are astaxanthin and canthaxanthin (Simpson et al., 2012). In the flesh of wild salmonids (salmon and trout), astaxanthin accounts for more than 90% of the total carotenoid content. Currently, commercial salmon diets contain either or both of the synthetic pigments available, astaxanthin and canthaxanthin. At equal flesh concentrations, astaxanthin results in a redder hue than canthaxanthin in salmon flesh (Buttle et al., 2001). In addition to colouring the fillet, carotenoids serve many biological functions for salmonids related to growth, reproduction and tissue health which is attributed to the compounds strong antioxidant properties (Anderson, 2001).

Fish metabolism of carotenoids is based solely on structural considerations which can include cleavage (both polyene chain and ionone ring) and chain shortening, hydroxylation/oxidation and dehydration (of xanthophylls) (Krinsky et al., 2004). Fish are able to metabolise carotenoids but this varies significantly between species and is dependent on the type of carotenoid available. Carotenoids are distributed unevenly in various tissues with higher concentration in liver and adipose tissue than in muscles (Decker et al., 2000). Factors influencing the absorption and deposition of carotenoids in Atlantic salmon are reviewed by Torrissen et al. (1989). Briefly the quantity available, carotenoid structure and the ability of salmon to metabolise or biotransform available carotenoids all have an impact on absorption and deposition of dietary carotenoids by the fish. Likewise factors such as fish size, sex, species and diet composition can impact subsequent deposition of carotenoids in muscles and tissues (Buttle et al., 2001; Hamre et al., 2004).

Atlantic salmon are generally poorer depositors of carotenoids and often the retention levels are lower compared to other fish species (Buttle et al., 2001; Chimsung et

al., 2012). Low absorption, catabolism in the liver, and high excretion are all major factors for the low retention of astaxanthin and canthaxanthin (Chimsung et al., 2012). Some aquatic species (i.e. *Penaeus japonicus* and *Portunus triuberculatus*) are able to transform ingested carotenoids such as β-carotene and zeaxanthin into astaxanthin; however Atlantic salmon are not able to perform such transformations (Shahidi & Brown, 1998; Torrissen et al., 1989). In fact, the opposite reduction has been confirmed in Atlantic salmon where dietary astaxanthin was reduced to zeaxanthin (Schiedt, 1985).

Synthetic pigments traditionally used in salmon feed continue to be utilized with success, however pigment alternatives should be considered with the increase in consumer preference for natural food additives. Some literature suggests the use of seaweed carotenoids in fish diets can influence flesh colour particularly in salmonid species. For example, the inclusion of *Porphyra dioica* in rainbow trout diets, effectively enhanced fillet colour as a result of yellow xanthophylls (zeaxanthin and lutein) (Soler-Vila et al., 2009). The use of natural colourants is of considerable interest to the organic salmon farming industry (Holdt & Kraan, 2011).

1.16.2 Supplementation of seaweed into porcine diets

In comparison to ruminants, pigs are considered monogastric animals and have a simple stomach where only slight microbial modifications of available nutrients take place before digestion and absorption occurs (Decker et al., 2000). As a result, seaweeds are not as commonly used in porcine diets as in cattle feed, however some research exist on the inclusion of low levels (<2%) to enhance pig health and pig meat quality (FAO, 2013). Supplementation of seaweed polysaccharides into porcine diets has proved beneficial in improving gut health and performance in pigs (Lynch et al., 2010a; O'Doherty et al., 2010).

Porcine dietary supplementation studies demonstrate that brown seaweed polysaccharides such as laminarin and fucoidan are resistant to digestion in the upper gastrointestinal tract, but stimulate the population growth of beneficial microbes in the intestine and subsequently improve the gut environment by enhancing the production of short chain fatty acids (Devillé et al., 2007; Lynch et al., 2010a, 2010b; Reilly et al., 2008; Scheppach, 1994; Smith et al., 2011). In weaning piglets, seaweed extracts provide a dietary means to improve gut health and subsequently reduce pathogen carriage in finishing pigs (Devillé et al., 2007; Dierick et al., 2010; Lynch et al., 2010b; Smith et al., 2011). Several studies have focused on the inclusion of laminarin and fucoidan, independently or in combination, on post weaning piglet performance and gut microflora. Porcine diets supplemented with the seaweed polysaccharides demonstrated an increased daily gain and gain to feed ratio. Laminarin and fucoidan significantly reduced *E. coli* populations and subsequently enhanced gut health of the pigs (McDonnell et al., 2010; O'Doherty et al., 2010).

1.17 Direct addition of seaweed / seaweed extracts in food systems

Seaweed and seaweed extracts directly added to food can increase the nutritional profile and serve as a functional ingredient by improving numerous technological properties (water holding capacity, water swelling capacity, water-solubility, fat binding capacity and viscosity). Additionally, the incorporation of dietary fibre with antioxidant compounds directly into food may enhance oxidative stability of the food during production and storage (Elleuch et al., 2011). Low levels (0.2-1.0%) of soluble fibres are commonly used for their functional properties while insoluble fibres are mainly used for their nutritional benefits. Mabeau & Fleurence (1993) have reported on various

biochemical and nutritional aspects of dietary fibre from seaweed sources used in food products.

1.17.1 Influence on quality and shelf-life parameters

Several quality and shelf-life parameters including colour, lipid oxidation, microbiological analysis, sensorial properties and texture can be influenced by the addition of ingredients such as seaweed extracts. Microbial growth, colour change and oxidative rancidity are the major problems which reduce the shelf-life of meat and meat products (Shan et al., 2009). Therefore, quality parameters are routinely monitored in shelf-life studies investigating the addition of bioactive ingredients.

1.17.1.1 Colour

The purchase of meat is more strongly influenced by colour than any other quality factor owing to the fact that consumers use discoloration as an indicator of freshness and quality (Mancini & Hunt, 2005). The concentration and chemical state of heme iron pigments, which are susceptible to oxidation, largely determines meat colour (Faustman & Cassens, 1990). Myoglobin which contains heme iron, can exist in one of three forms: deoxymyoglobin (purplish-red), oxymyoglobin (cherry-red), or metmyoglobin (brown) in intact muscle (Cruz et al., 2014). Myoglobin and lipid oxidation have been reported to occur in a concurrent manner in muscle foods, with each process having the ability to influence the other (Chaijan, 2008). Mancini and Hunt (2005) have extensively reviewed the various factors which influence meat colour and colour stability.

1.17.1.2 Lipid oxidation

Lipid oxidation is a key deteriorative process in muscle foods (O'Grady & Kerry, 2009). The rate and extent of lipid oxidation in muscle foods is influenced by pre and post-slaughter factors including stress, diet, carcass temperature and post-mortem pH decline (Morrissey et al., 1998). Many review articles exist on the subject of lipid oxidation in muscle foods (Chaijan, 2008; Decker & Xu, 1998; Faustman & Cassens, 1990; Ladikos & Lougovois, 1990). Much research into ways of minimising or delaying lipid oxidation has been carried out, including the use of supplements in animal diets (Jiménez-Colmenero, et al., 2001). The meat industry has successfully used synthetic antioxidants to retard the rate of lipid oxidation in muscle foods (Coronado et al., 2002). The effect of antioxidants on the oxidation of foods depends on concentration, their polarity and the presence of other antioxidants (Choe & Min, 2009).

1.17.1.3 Microbiological analysis

Antimicrobials are often employed in meat products to extend shelf-life and control pathogens. *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella enterica* are common foodborne pathogenic bacteria frequently isolated in meat products. The spoilage and poisoning of food due to the growth of these and other bacteria can result in meat unfit for human consumption (Holley et al., 2004). Increased bacterial growth is also known to negatively affect the colour of meat. For centuries, dietary herbs and spices have been used successfully as natural food additives to extend shelf-life by reducing or eliminating survival of these and other pathogenic bacteria (Shan et al., 2009).

1.17.1.4 Sensory analysis

Sensorial properties can be greatly influenced by the addition of functional ingredients due to the characteristically strong odours and flavours they impart. The flavour profile generally determines consumer acceptability of the meat. Odour and taste are the main contributors to flavour (Melton, 1990). Meat flavour can be influenced by feed source and genetics of the animal. The major precursors of meat flavour can be divided into two categories: water-soluble components and lipids. The main water-soluble flavour precursors are suggested to be free sugars, sugar phosphates, nucleotide bound sugars, free amino acids, peptides, nucleotides and other nitrogenous components (Shahidi et al., 1986). The characteristic flavour of meat develops from reactions occurring during heating, principally the Maillard reaction and the degradation of lipids. Both type of reactions produce a wide range of products, including a large number of volatile compounds found in cooked meat (Mottram, 1998).

1.17.1.5 Texture

Texture is one of the most important sensory attributes of meat, after appearance and flavour. Commonly, instrumental texture assessment on meat is performed using a texturometer device which allows tissue resistance both to shearing and to compression to be measured. Texture includes a complex variety of measurable characteristics including: hardness (toughness), springiness, cohesiveness, gumminess and chewiness (juiciness) (de Huidobro et al., 2005). Textural and stabilizing properties of seaweed extracts in food products are strongly related to the hydration properties of the polysaccharides. Hydration properties are measured by four parameters: water holding capacity, water binding capacity, swelling and solubility. Water holding capacity and water binding capacity parameters are only relevant for insoluble fibres (Rupérez & Saura-Calixto,

2001). A positive correlation between water retention and swelling capacity has previously been observed in seaweeds. In general, brown seaweeds have higher swelling and water retention capacity than red seaweeds (Rupérez & Saura-Calixto, 2001).

1.17.2 Functionality of seaweed / seaweed extracts in muscle based food systems

Previously, dietary fibres from seaweed have been investigated in the formulation of different meat products with the aim to improve texture, increase cooking yields and reduce formulation costs (Cofrades et al., 2008). Many researchers have demonstrated that low-fat meat products with added dietary fibre, from seaweeds, have improved textural properties (Cofrades et al., 2008; Fernández-Martín et al., 2009; López-López et al., 2009a). For example Choi (2012) added *Laminaria japonica* powder to reduced-fat pork patties, without any detrimental effects on meat quality, as perceived by the consumer. The reduced-fat pork patties containing the *Laminaria japonica* powder also had improved quality characteristics that were similar to the control patties containing twice the fat content. Seaweed fibre has been used as a means to overcome the technological problems associated with low-salt meat products. The use of seaweed has improved fat and water binding properties of low-salt meat products (Cofrades et al., 2011). Table 1.16 contains a summary of the impact of seaweeds / extracts on quality parameters of processed meat products.

1.18 Stability of seaweed / seaweed extracts in meat products: influence of processing

For the successful use of seaweed and/or seaweed extracts in cooked meat products, it is important to understand their molecular properties, interactions with food ingredients as well as the influence of processing conditions. The stability of seaweed

Table 1.16. The use of seaweed/seaweed extract in muscle based foods/systems.

Model system	Seaweed/seaweed extract	Highlights	Reference
Pork meat batter/emulsion	*Himanthalia elongata* (3.4%)	-improved emulsion stability -prevented thermal denaturation of a considerable protein fraction	Fernández-Martín et al. (2009).
Low-salt and low-fat beef patties	*Undaria pinnatifida* (3%)	-lowered moisture contents -less thawing and cooking losses -did not negatively affect the sensory properties	López-López et al. (2010).
Low-fat frankfurters	*Himanthalia elongata* (5%)	-improved water and fat binding properties -increased the hardness and chewiness -reduced the springiness and cohesiveness -special flavour of seaweed resulted in less acceptable products	López-López et al. (2009b).
Reduced/low-fat, low-salt frankfurters	*Himanthalia elongata* (3.3%)	-increased cooking loss and reduced emulsion stability -formation of a more heterogeneous structure -no significant difference on texture sensory but lower overall acceptability because of an seaweed-like off-flavour	Jiménez-Colmenero et al. (2010).
Low-salt frankfurters	*Himanthalia elongata* (5%)	-improved lipid profile of frankfurters -frankfurters were Ca-rich, low-sodium with better Na/K ratios and added fibre	López-López et al. (2009c).
Low-salt restructured poultry	*Himanthalia elongata* (3%)	-increase in purge loss but reduced cooking loss -texture values with seaweed were the same as the control -increased TVC due to the seaweed -decreased the lightness and redness and increased the yellowness	Cofrades et al. (2011).

Table 1.16 (*continued*). The use of seaweed/seaweed extract in muscle based foods/systems

Model system	Seaweed/seaweed extract	Highlights	Reference
Reduced-fat pork patties	*Laminaria japonica* (1, 3 and 5%)	-higher moisture, ash, carbohydrate content, yellowness and springiness -enhanced overall acceptability at 1 and 3% addition -improved quality characteristics to acceptable levels	Choi et al. (2012).
Low-salt pork meat emulsion model systems	*Himanthalia elongata* (5.6%) *Undaria pinnatifida* (5.6%) *Porphyra umbilicalis* (5.6%)	-increased n-3 polyunsaturated fatty acids and decreased n-6/n-3 PUFA ratio -increased the antioxidant capacity due to soluble polyphenolic compounds	López-López et al. (2009a).
Low-salt gel/emulsion pork meat systems	*Himanthalia elongata* (2.5 and 5%) *Undaria pinnatifida* (2.5 and 5%) *Porphyra umbilicalis* (2.5 and 5%)	-improved ($P < 0.05$) water- and fat-binding properties -increased hardness and chewiness, lowered values for springiness and cohesiveness -reduced a* values of gel/emulsion system	Cofrades et al. (2008).

polysaccharides during processing is an important consideration as many carbohydrates are susceptible to changes during heat treatment at 100°C or higher (Venugopal, 2011). Cooked meat is susceptible to oxidation and influenced by a larger number of factors than fresh meat due to thermal processing.

The potential for non-enzymatic browning reactions (Maillard reactions) to occur between reducing sugars present at the end of seaweed polysaccharide chains and free amino groups, peptides or proteins in meat products during processing and storage is also present. These reactions are dependent on temperature and readily happen with intermediate water activity (Venugopal, 2011). Nicoli et al. (1999) reported that the

overall antioxidant capacity of certain foods may be enhanced due to improvement in the antioxidant properties of naturally occurring antioxidants and the formation of Maillard reaction products (MRPs). MRPs have been linked to beneficial properties such as antimutagenic, antimicrobial and antioxidative activities. In foods, MRPs have had antioxidant effects on honey and tomato purees (Anese et al., 2010; Antony et al., 2000). The addition of honey to turkey breast prior to heating had an antioxidant effect on the meat which was attributed to MRPs formed during heating (Antony et al., 2000). Radical scavenging activity of dried *Petalonia binghamiae* extracts was increased after being heated for 1 hour at 121°C. The content of brown pigments after heating was increased approximately 2.5 fold and was attributed to the generation of MRPs during heating which subsequently enhanced the radical scavenging activity of the extract (Kuda et al., 2006)

Many antioxidants can be sensitive to heat treatments, however limited studies exist which investigate the effect of heat on antioxidant compounds from seaweeds in food systems. In one study, cooked pasta with dried seaweed (*Undaria pinnatifida*) at 4 levels (5-30%) resulted in lower levels of phenolic compounds and reduced DPPH activity compared to the uncooked pasta. However antioxidant activity of the cooked seaweed pasta was significantly higher than the control, suggesting some antioxidant compounds were present in the pasta after thermal processing. Additionally the reduction of fucoxanthin and fucosterol in the pasta was less than 10%, indicating thermal stability of antioxidant compounds in a gluten based maxtrix (Prabhasankar et al., 2009). Similarly, thermal stability at 100°C of hydrogen peroxide scavenging activity of brown seaweed (*S. horneri* and *E. cava*) extracts has been reported (Heo et al., 2005).

1.18.1 Bioaccessibility and bioavailability of bioactive ingredients

To adequately assess the beneficial effects of seaweed compounds with antioxidant activity - bioaccessibility, bioavailability and bioactivity post digestion need to be established. Determining bioaccessibility and bioavailability can be difficult to conduct and monitor in human studies (*in vivo*), therefore *in vitro* models are often applied such as digestion models and human epithelial cell cultures (Grajek et al., 2005).

Bioaccessibility is defined as the fraction of the compound transferred from the food matrix during digestion and thus made accessible for intestinal absorption (Carbonell-Capella et al., 2014). The bioavailability of a compound is dependent on stability post cooking and the stability through the gastrointestinal tract (GIT), absorption through the intestinal walls into the blood stream and subsequent availability for use in metabolic functions (Rafiquzzaman et al., 2013). Bioactivity refers to the phenomena that occur after the component has reached the systemic circulation, is transported to relevant tissues, interacts with biomolecules, is metabolised and all the cascade of physiological effects it generates. Bioactivity can be assessed *in vitro* or *in vivo*, however caution must be exercised in using very specific methodologies for *in vitro* studies in order for results to correlate with *in vivo* studies. Likewise, *in vivo* studies should be carried out whenever possible to validate *in vitro* methods (Cardoso et al., 2015).

With concerns regarding metallic contamination of seaweeds and toxicity levels of minerals, a limited number of *in vitro* digestion studies have investigated the bioavailability of arsenic, cadmium, mercury, lead and iodine in seaweeds (Cardoso et al., 2015; Gonzalez et al., 2011). To date, however, no studies exist analysing the bioactivity of seaweed compounds incorporated in muscle based foods, post digestion.

1.18.2 Functional properties of seaweed polysaccharides as dietary fibre

The use of seaweeds as dietary fibre is discussed briefly. Seaweed polysaccharides are considered a source of dietary fibre with prebiotic potential and other functionalities owing to the fact a large amount of seaweed polysaccharides cannot be digested completely by the human digestive system (Kim, 2012). Dietary fibre is a complex material consisting of plant cell walls, structurally complex and chemically diverse polysaccharides and other associated substances (Venugopal, 2008). The diversity of structure and composition of dietary fibres makes it difficult to elucidate their mechanisms of action (Thebaudin et al., 1997). Dietary fibre (25–75% DM) of seaweeds differs chemically and physicochemically from the fibre of terrestrial plants and may offer other physiological effects (Rupérez & Saura-Calixto, 2001). The structure as well as the physiochemical and biological properties of seaweed dietary fibre has been reviewed previously (Lahaye & Kaeffer, 1997; Mišurcová et al., 2012). A summary of seaweed dietary fibres is presented in Table 1.17.

Table 1.17. Dietary fibres from seaweed sources.

Fibre type	Seaweed constituents[ab]	Activity during digestion[c]
Soluble fibre (50-85%)	agars, alginic acid, fucoidan, laminarin and porphyran	Slowing down digestion and absorption of nutrients by increasing viscosity through the gastrointestinal tract but do not markedly change colonic function.
Insoluble fibre (12-40%)	cellulose, lignins, mannans and xylans	Decrease intestinal retention time and increase stool weight.

[a]O'Sullivan et al. (2010).
[b]Rupérez & Saura-Calixto (2001).
[c]Thebaudin et al. (1997).

Dietary fibres may act as fermentative substrates which modify the activity of gut microflora and result in modification or reduction in the production of mutagens. The consumption of seaweed polysaccharides as a dietary fibre source has been linked to the growth and protection of beneficial intestinal microflora, thereby improving the overall

health of the intestines of humans and animals alike (Gupta & Abu-Ghannam, 2011b). O'Sullivan et al. (2010) reviews the potential for seaweed polysaccharides to be used as prebiotics in human and animal health applications with a discussion of both *in vitro* and *in vivo* studies. A systematic review of immunomodulatory activities of dietary polysaccharides is presented by Ramberg et al. (2010).

Dietary fibre is not a single compound but a combination of chemical substances with varied composition and structure (Grajek et al., 2005). Dietary fibre can act as a carrier of antioxidants some of which are resistant to gastric degradation and released from the fibre matrix in the colon (Saura-Calixto, 2010). Phytochemicals having antioxidant activity associated with dietary fibre include polyphenols, carotenoids, sterols, lignans, terpenoids and sulphated polysaccharides. These so-called co-travellers of dietary fibre reach the intestinal tract as nondigestible food and may offer additional benefit to the host (Jiménez-Escrig et al., 2012). Seaweed soluble fibres are a source of important antioxidant compounds including sulphated polysaccharides (Elleuch et al., 2011; Venugopal, 2008). Functional and nutritional properties associated with dietary fibre include the antioxidant activity of these associated compounds which can act as free radical scavengers through the entire digestive tract and in colonic tissues (Esposito et al., 2005). The role of dietary fibre as an antioxidant carrier is well reviewed by Saura-Calixto (2010).

1.18.3 Transepithelial transport and cellular uptake of bioactivity

Digestion simulation is considered the first step in the evaluation of bioaccessibility and intestinal absorption and the metabolic transformation particularly in the hepatic tissues also needs to be considered. Digested samples can be transferred to an *in vitro* cell culture model (O'Callaghan & O'Brien, 2010). Typically, cultures of human

colorectal cancer epithelial cell lines (Caco-2) and a transwell assay are utilised to mimic the intestinal lining barrier. Caco-2 cells are grown on a porous polyethylene terephthalate membrane in a 12 well format to form monolayers of polarized cells possessing function similar to intestinal enterocytes (Yee, 1997). Likewise, Caco-2 cells have the capacity to carry out metabolic transformations (Cardoso et al., 2015). This model has been previously used to examine the transport and cellular uptake of a number of plant extracts (i.e. rosemary, sage and Echinacea) and isolated compounds (i.e. carotenoids and α-tocopherol) (O'Callaghan & O'Brien, 2010). Previously reported studies indicate a strong correlation between *in vitro* Caco-2 cellular uptake and transport of a variety of compounds and *in vivo* human absorption (Yee, 1997).

1.19 Seaweed industry in Ireland

Ireland is an island with a 7,000 kilometre coastline in the fertile waters of the Gulf Stream full of marine organisms including an abundance of seaweed species. Industrial potential including high-value applications of sustainable resources of seaweed has not been fully realised (Sea Change, 2006). Utilising the abundance of seaweed off the coasts of Ireland is now the focus of a major study in functional foods research. Irish seaweeds, like other seaweeds distributed about the globe, are an ideal source of ingredients for use in functional foods.

The Irish seaweed industry consists of approximately 18 small to medium sized enterprises at a small number of licensed sites for harvesting from Co. Donegal to Co. Waterford. Approximately 700 people are employed in either a full-time, part-time or seasonal basis, mostly in remote rural areas of the west coast primarily within the Gaeltacht (Sea Change, 2006). Biopolymers and agriculture/horticulture are the two most economically important sectors for Ireland's seaweed industry (Guiry, 2014). Traditional

hand harvesting of *Ascophyllum nodosum* for alginate extraction and agriculture/horticulture applications sustains the industry, especially along the west coast of Ireland (Werner et al., 2004). In the last fifteen years, a few companies have initiated production and commercialization of higher value products from Irish seaweeds (Morrissey et al., 2001). According to Sea Change Strategy (2006), the Irish seaweed production and processing sector will be worth €30 million per annum by 2020.

Irish food products with added seaweed – Cannelloni with salmon and seaweed, Brown bread with seaweed, Cheddar cheese with seaweed

Over 500 species (80 green, 271 red and 147 brown) of seaweed grow in Irish waters (Guiry & Guiry, 2014). About 16 seaweed species are commercially utilised in Ireland, however *A. nodosum*, and two species of red calcified coralline seaweed, referred to as Maërl (*Phymatolithon calcareum* and *Lithothamnion corallioides*) dominate most of Ireland's production (Werner et al., 2004). Other species of seaweeds harvested in smaller quantities in Ireland for use as food include dulse, carrageen moss and various kelps and wracks. The human food and cosmetic sector consists mainly of smaller business's which employ on average 5 people or less, with very little automation in harvesting, drying or processing (Sea Change, 2006). A list of Irish suppliers of seaweed and / or products that contain seaweed ingredients is compiled in Table 1.18.

Table 1.18. Irish suppliers of seaweed and / or products that contain seaweed ingredients

Supplier	Sector
Arramara Teo[a]	Seaweed meal
AlgAran[a]	Seaweed meal, edible seaweed, cosmetics
Irish Seaweed processors[a]	Edible seaweed
Kinsale Gourmet[a]	Edible seaweed
Spanish Point Sea Vegetables[a]	Edible seaweed
LoTide Fine Foods[b]	Edible seaweed
Roaringwater Bay Seaweed co-op[b]	Edible seaweed
Blath na Mara[b]	Edible seaweed, cosmetics
Rí na Mara[a]	Cosmetics
SeaVite[a]	Cosmetics
Voya[a]	Cosmetics
Carabay Seaweed Health Products[b]	Cosmetics
Brandon Products[a]	Liquid seaweed products
Sea Nymph[a]	Liquid seaweed products
Oilean Glas[a]	Liquid seaweed products
Marrigot[b]	Functional marine materials
BioAtlantis[b]	Agriculture, feed supplements, human nutraceuticals
Ocean Harvest Ireland[a]	Agriculture, aquaculture feeds, feed supplements
Cybercolloids[b]	Hydrocolloid applications in food and non-food products

[a]Guiry & Guiry (2014).
[b]Rhatigan (2009).

1.20 Conclusion

This review endeavours to present the state-of-the-art in terms of the use of macroalgae (seaweed) and macroalgal polysaccharides as functional ingredients in muscle foods. The composition of macroalgae is extensively reviewed and bioactivity present in each species highlighted (*Ulva rigida*, *Palmaria palmata* and *Laminaria digitata*). The potential of muscle foods (fish and pork meat) as suitable vehicles/carries of functional ingredients is also investigated.

1.21 Thesis Objectives

The ultimate aim of this thesis was to evaluate the use of macroalgae and commercial macroalgal polysaccharides, rich in compounds with natural biological activity, in muscle based foods.

The studies in this thesis were undertaken with the following objectives:

- To evaluate the effects of different levels (0-15%) of macroalgae (*Ulva rigida* and *Palmaria palmata*) in farmed Atlantic salmon (*Salmo salar*) diets on shelf-life and sensory parameters of the salmon fillets.
- To examine porcine dietary supplementation of commercially sourced wet and spray-dried macroalgal extracts containing polysaccharides (laminarin and fucoidan), from brown seaweed (*Laminaria digitata*), on the quality and shelf-life of fresh pork steaks.
- To investigate the effects of dietary laminarin and fucoidan levels, form and duration of feeding on the quality and shelf-life of fresh pork.
- To evaluate the efficacy of the spray-dried extract containing laminarin and fucoidan added directly to pork meat on quality and shelf-life parameters of fresh and cooked pork patties.
- To determine the bioaccessibility of macroalgae-derived bioactive components in cooked pork meat using an *in vitro* digestion procedure coupled with Caco-2 cell model.

Atlantic salmon studies

CHAPTER 2

Influence of green seaweed (*Ulva Rigida*) supplementation on the quality and shelf-life of Atlantic salmon fillets

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ABSTRACT

Supplementation of salmon (*Salmo salar*) diets with *Ulva rigida* (UR) (0, 5, 10 and 15% UR) or synthetic astaxanthin (positive control, PC) for 19 weeks pre-slaughter on quality indices of fresh (raw) salmon fillets was examined. Susceptibility of salmon fillets/homogenates to oxidative stress conditions (cooking/iron-ascorbate induced oxidation) was also measured. In salmon fillets stored in modified atmosphere packs (60% N_2 : 40% CO_2) (MAP) for up to 15 days at 4ºC, *U. rigida* increased surface '-a*' greenness and 'b*' yellowness values in a dose-dependent manner resulting in a final yellow/orange flesh colour. Proximate composition, pH and lipid oxidation (fresh, cooked and fillet homogenates) were unaffected by dietary addition of *U. rigida*. On day 12, 5% UR psychrotrophic bacterial growth was lower than controls. Feeding salmon 5% UR did not influence 'eating quality' sensory descriptors (texture, odour, oxidation flavour and overall acceptability) in cooked salmon fillets compared to 0% UR. Higher levels of dietary *U. rigida* (10 and 15% UR) were negatively correlated with colour and overall acceptability descriptors. Results indicated that dietary *U. rigida*, at a level of 5%, may prove to be a functional ingredient in salmon feed to enhance salmon fillet quality.

2.1 INTRODUCTION

Macroalgae grow in abundance in coastal areas around the world and are classified into green (*Chlorophyta*), red (*Rhodophyta*), and brown (*Phaeophyceae*) seaweeds based on their chemical composition (Gupta and Abu-Ghannam, 2011a). Green seaweed, belonging to the genus *Ulva* generally grow in shallow waters and tide pools and are exclusively found in marine waters (Loughnane et al., 2008). Many species of *Ulva* (sea lettuce) are similar and difficult to differentiate with only seven species reported in Ireland and Britain: *Ulva rigida*, *U. lactuca*, *U. scandinavica*, *U. olivascens*, *U. gigantea*, *U. rotundata*, and *U. californica* (Hasan and Chakrabarti, 2009; Loughnane et al., 2008). *Ulva spp.* are a natural source of many bioactive compounds such as polysaccharides including ulvan (the main water soluble storage polysaccharide), protein, essential fatty acids, vitamins, minerals, carotenoids, polyphenols, etc. (Fleurence, 1999).

The use of nutrient rich seaweed as a natural alternative to fish meal has been investigated previously. A move towards partial substitution of fish meal by plant proteins is widely accepted within the aquaculture industry in an effort to reduce production costs (Lahaye and Robic, 2007). Early fish growth studies confirm the inclusion of 10-20% algae or seaweed meal is an acceptable fishmeal replacer in many fish diets without compromising fish growth or digestive efficiency (Hasan and Chakrabarti, 2009). Many species of *Ulva* (*Ulva rigida*, *U. oxysperma*, *U. lactuca*, *U. fasciata*, *U. reticulata*, *U. pertusa*) have been examined as ingredients (inclusion at 3-15%) in diets of fish including red sea bream (*Pagrus major*), Japanese flounder (*Paralichthys olivaceus*), yellowtail (*Seriola quinqueradiata*), nibbler (*Girella punctata*), European sea bass (*Dicentrarchus labrax*) and Nile tilapia (*Oreochromis niloticus*) resulting in enhanced growth rates, protein assimilation, immune function and lipid metabolism (Marinho et al., 2013; Mustafa et al., 1995; Nakagawa and Montgomery,

2007; Valente et al., 2006). Atlantic salmon (*Salmo salar*) diets containing *Ulva spp.* have been linked to improved resistance of fish to stress and disease (Mustafa and Nakagawa 1995; Satoh et al., 1987).

Due to the biological activities of seaweed constituents, dietary addition of *Ulva spp*. to farmed Atlantic salmon feed may result in a health promoting functional feed which surpasses basic nutritional requirements (Tacchi et al., 2011). Vitamins and minerals from *Ulva spp*. such as vitamins B_{12}, C and E, calcium, magnesium, iron and manganese are sufficient for Atlantic salmon nutrition (Burton et al., 2009; Chandini et al., 2008). The addition of antioxidants (water soluble vitamin C (ascorbic acid) and lipid soluble vitamin E (α-tocopherol)) to Atlantic salmon diets is important in early stage fish development and has been reviewed previously (Hardie et al., 1991; Waagbø, 1994). Salmon lack the ability to synthesize carotenoids and wild salmon feed on carotenoid containing crustaceans resulting in a pink flesh colouration. Therefore, farmed salmon diets are supplemented with pigments (astaxanthin (red) and canthaxanthin (orange)) in order to pigment the flesh at considerable economic expense (10 to 15% of total feed costs) (Torrissen et al., 1989). *Ulva spp*. contains a range of colour pigments (chlorophyll *a* and *b*, β-carotene, lutein, violaxanthin, antheraxanthin, zeaxanthin and neoxanthin) which may be a source of natural pigments for fish feed (Lobban and Wynne, 1981; Tacchi et al., 2011). Zavodnik (1987) reported the carotenoid content of *U. rigida* varied during the growing period (January to June) and was greatly influenced by local environmental factors and the season of harvest.

In addition to health and nutritional benefits, bioactive compounds in green seaweeds are a potential resource of many functional ingredients that may enhance fillet quality. Studies to assess the antioxidant activity of ulvan and its oligosaccharides have reported that they possess reducing power, free radical scavenging and metal chelating

activities usually due to the presence of sulphated groups (Alves et al., 2013; Chiellini and Morelli, 2011; Lahaye and Robic, 2007). Vitamins and vitamin precursors in green seaweeds, including α-tocopherols, niacin, thiamine and ascorbic acid may also demonstrate antioxidant activity (Chandini et al., 2008). *Ulva lactuca* has been reported to possess antioxidant and antibacterial activity due to carotenoids, chlorophyll derived and phenolic compounds (El Baky et al., 2009).

The nutritional value of seaweed included in salmon feed is generally evaluated by growth and survival of the fish, however the efficacy of seaweed to enhance fillet quality, shelf-life and consumer acceptability is frequently not determined. Therefore, Atlantic salmon diets enriched with green seaweed as a source of bioactive compounds to enhance salmon fillet quality merits investigation. The objective of this study was to examine the effect of including *Ulva rigida* (0-15%) in farmed salmon diets on the quality indices (composition, muscle pH, colour, lipid oxidation, microbiology and sensory properties) of salmon fillets. Susceptibility of salmon fillet homogenates to iron-ascorbate induced lipid oxidation was also investigated.

2.2 MATERIALS AND METHODS

2.2.1 Reagents

All chemicals used were 'AnalaR' grade and were obtained from Sigma-aldrich Chemie GmbH, Steinheim, Germany; Oxoid Ltd., Basingstoke, Hampshire, England; Merck KGaA, Darmstadt, Germany. *U. rigida* was harvested during May to July 2011 from the coasts of Harbour View Bay, Cork, Ireland.

2.2.2 Salmon and diets

After harvesting, *U. rigida* was subsequently washed and dried (at 40°C using a dehumidifying oven) before addition to the salmon diet formulations. Diets were prepared at the feed formulation laboratory in Carna Research Station, Ryan Institute, National University of Ireland, Galway. All diets were formulated to be *iso*-nitrogenous (40%), *iso*-lipidic (25%) and *iso*-caloric (26 MJ kg^{-1}). The composition of the experimental diets is outlined in Table 2.1.

Table 2.1. Composition of the experimental diets with inclusion of *Ulva rigida* (g kg^{-1}, unless otherwise indicated).

	PC	**0% UR**	**5% UR**	**10% UR**	**15% UR**
Fish meal[a]	407.4	407.4	390.8	374.1	357.5
Fish oil[a]	200.0	200.0	201.4	202.8	204.1
Ulva rigida	-	-	50.0	100.0	150.0
Corn starch[b]	187.3	187.6	152.8	118.1	83.4
Lysamine[c]	90.0	90.0	90.0	90.0	90.0
Glutalys[c]	90.0	90.0	90.0	90.0	90.0
Mineral & vitamin premix[d]	20.0	20.0	20.0	20.0	20.0
Lucantin Pink[e]	0.30	-	-	-	-
Barox Plus[f]	5.00	5.00	5.00	5.00	5.00

[a]United fish products Ltd., Donegal, Ireland.
[b]Laboratory grade, Sigma –Aldrich Company Ltd., Poole, UK.
[c]Purified feed ingredients, Roquette, France.
[d]Premier nutrition products Ltd., Staffordshire, UK. (Manufacturers analysis: Ca 11.50%, Ash 78.71%, Na 8.86%, Vitamin A 1.0µg/kg, Vitamin D3 0.10%, Vitamin E 7.0 g/kg, Cu 250 mg/kg, Mg 15.6 g/kg and P 6.4 g/kg).
[e]BASF, Ludwigshafen, Germany.
[f]Kemin Europa N.V., Herentals, Belgium.

Atlantic salmon (*Salmo salar*) smolts were obtained from a commercial company based at Lough Fee, Connemara, Ireland. After a 10 week acclimation period, a 19-week completely randomised experimental feed trial was carried out at the Carna Research Station. Salmon (n = 35, average fish weight ~ 174.6g) were randomly assigned to one of fifteen tanks (three tanks per treatment) and fed one of five experimental diets. The positive control group (PC) were fed a basal diet plus synthetic astaxanthin (0.3g/kg feed) (Table 2.1). The remaining four groups were fed the basal diet plus *U. rigida* (UR) at 0 (0% UR), 5 (5% UR), 10 (10% UR) and 15% (15% UR) inclusion levels. The salmon were hand-fed on five occasions over the course of each day and housed in 1000 L tanks fed by a filtered flow-through seawater supply (ambient temperature regime). At the end of the feeding trial, salmon (average fish weight ~ 521.50 g), were euthanised with a sharp blow to the head followed by the pithing of the brain and were gutted. Fresh (raw) salmon fillets were transported on ice at 4ºC to the School of Food and Nutritional Sciences at University College Cork, Ireland.

2.2.3 Proximate analysis of fresh salmon fillets

The proximate composition of fresh salmon fillets was reported on a wet weight basis. Protein (nitrogen x 6.25) was determined by the Kjeldahl method of the Association of Official Analytical Chemists (AOAC, 1995). The moisture and fat content were measured using the SMART Trac rapid moisture/fat analyser (CEM Corporation, NC, USA). The ash content was determined using a muffle furnace (AOAC 1995). Compositional analysis results were expressed as percentage values, %.

2.2.4 Salmon processing and packaging

Fresh salmon fillets (~100 g) (PC, 0, 5, 10 and 15% UR) were placed in low oxygen permeable (<1cm^3/m^2/24 hr at STP) polystyrene/ethylvinylalcohol/polyethylene trays. Trays were covered with a low oxygen permeable (3 cm^3/m^2/24 hr at STP) laminated barrier film with a polyolefin heat-sealable layer. Using modified atmosphere packaging technology, the trays were flushed with 60% N_2 : 40% CO_2 using a vacuum-sealing unit (VS 100, Gustav Müller and Co. KG, Bad Homburg, Germany) equipped with a gas mixer (Witt-Gasetechnik GmbH and Co. KG, Witten, Germany) and heat-sealed. Fresh salmon fillets were stored for up to 15 days under fluorescent lighting conditions (approximately 660 lux) at 4°C. The gas atmosphere (% O_2 and % CO_2) in the modified atmosphere packs (MAP) was checked using a CheckMate 9900 (PBI-DanSensor, Denmark). Immediately after gas flushing, MAP contained 57.86 ± 0.11% N_2 and 42.00 ± 0.14% CO_2. The average gas composition in MAP at the end of the 15 day storage period was 57.49 ± 0.49% N_2 and 42.51 ± 0.42% CO_2. Percent nitrogen was calculated by difference of % O_2 and % CO_2.

In a cooked fish study, salmon fillets from all treatments (PC, 0, 5, 10 and 15% UR) were placed on aluminium foil lined trays and cooked at 180°C for 12 min in a fan-assisted convection oven (Zanussi Professional, Model 10 GN1/1, Conegliano, Italy) until an internal temperature of 72°C was reached. Cooked fillets were placed in trays over-wrapped with oxygen permeable film and stored aerobically for up to 5 days at 4°C.

2.2.5 Measurement of pH

Fresh salmon fillet samples (10 g) were homogenised for 1 min at 24,000 rpm in 90 ml distilled water using an Ultra Turrax T25 homogeniser (Janke and Kunkel, IKA-Labortechnik, GmbH and Co., Staufen, Germany) and the pH was measured at 20°C

using a pH meter (Seven Easy portable, Mettler-Toledo GmbH, Schweizenbach, Switzerland). The pH of salmon fillets was recorded on days 1, 3, 7, 12 and 15 of storage.

2.2.6 Colour measurement

The surface colour was measured using a Konica Minolta CR-400 Chroma-Meter (Minolta Camera Co., Osaka, 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 on the CIE LAB colour space system using a white tile (D_c:L = 97.79, a = -0.11, b = 2.69). The 'L*' value represents lightness and 'a*' and 'b*' values represent redness and yellowness, respectively. Colour measurements of fresh salmon fillets were recorded on days 1, 3, 7, 12 and 15.

2.2.7 Measurement of lipid oxidation

Lipid oxidation was measured using the 2-thiobarbituric acid assay as described by Siu and Draper (1978). Chopped salmon fillet samples (5 g) were homogenised for 2 min in 25 ml distilled water using an Ultra Turrax T25 homogeniser (Janke and Kunkel, IKA-Labortechnik, GmbH and Co., Staufen, Germany). Trichloroacetic acid (10%) was added (25 ml) and the mixture was shaken vigorously and filtered through Whatman No. 1 filter paper. In screw capped test tubes, 4 ml of clear filtrate was added to 1 ml of 0.06 M 2-thiobarbituric acid (TBA). The tubes were placed in a water bath and held at 80°C for 90 min. The absorbance of the filtrate was measured spectrophotometrically (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 of 0.06 M TBA reagent). The malondialdehyde content of the sample was calculated using an

extinction coefficient of 1.56 x 10^5 M^{-1} cm^{-1}. Results were expressed as 2-thiobarbituric acid-reactive substances (TBARS) in mg malondialdehyde (MDA)/kg salmon. Lipid oxidation in fresh salmon fillets was measured on days 1, 3, 7, 12 and 15 of storage and on days 0, 1, 3 and 5 in cooked salmon fillets.

2.2.7.1 Preparation of salmon fillet homogenates

Fresh salmon fillet homogenates (25%) were prepared by homogenising 25 g tissue in 75 ml 0.12M KCl 5 mM histidine (pH 5.5) using an Ultra Turrax T25 homogeniser at 24,000 rpm for 3 min. Stock solutions of ferric chloride ($FeCl_3$) (4500 μM) and sodium ascorbate (4500 μM) were freshly prepared each day in distilled water. 0.3 ml of both were added to 29.4 ml homogenate thus diluting both 1/100 (i.e. 0.3 ml of each in a total volume of 30 ml) to achieve final concentrations of 45 μM for both $FeCl_3$ and sodium ascorbate. Lipid oxidation in salmon fillet homogenate incubates (30 ml) was initiated with equimolar ferric chloride ($FeCl_3$) : sodium ascorbate (45 μM). Homogenates without $FeCl_3$ and ascorbic acid were run simultaneously as controls. Lipid oxidation in fresh salmon homogenates was measured immediately (time 0) and after 1, 4 and 24 hrs storage at 4°C.

2.2.7.2 Measurement of lipid oxidation in salmon fillet homogenates

A modification of the 2-thiobarbituric acid (TBA) assay of Siu and Draper (1978) was used to measure lipid oxidation in fresh salmon fillet homogenates. Homogenate samples (4 ml) were added to 4 ml 10% trichloroacetic acid (TCA). The samples were mixed using a vortex mixer and the precipitate formed was removed by filtering through Whatman No. 1 filter paper. In a screw cap test tube, 4 ml of clear supernatant was added to 1 ml 0.06 M 2-thobarbituric acid (TBA). The tubes were incubated at 80°C for 90 min

and the absorbance of the resulting coloured complex was measured using a spectrophotometer at 532 nm against a blank containing all reagents and distilled water instead of the filtrate. The malondialdehyde content was calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. Results were expressed as TBA reactive substances (TBARS) in mg malondialdehyde (MDA)/kg salmon.

2.2.8 Microbiological analysis

Fresh salmon fillet samples (10 g) were transferred into stomacher bags, diluted with 90 ml of maximum recovery diluent and stomached for 3 min (Steward Stomacher 400 Lab Blender, London, UK) resulting in a 10^{-1} dilution used for analysis. Serial dilutions were prepared and 0.1 ml aliquots from each dilution were plated onto standard plate count agar (PCA) (Oxoid Ltd.). The plates were incubated at 30°C for 48 hr and at 4°C for 10 days to determine mesophilic and psychrotrophic counts, respectively. Microbiological analysis of fresh salmon fillets was carried out on days 1, 3, 7, 12 and 15 of storage. Results were expressed as $\log_{10}$CFU (colony forming units)/g salmon.

2.2.9 Sensory evaluation

Sensory analysis ('visual' and 'eating quality') of fresh and cooked salmon fillets stored in 60% N_2 : 40% CO_2 was performed in duplicate by 26 naïve assessors on days 1 and 7 of storage following the method of O'Sullivan et al., (2003). On day 12, the bacterial count was deemed unsafe to continue further sensory evaluation. 'Visual' sensory analysis descriptors examined were: pinkness, whiteness, drip, purchasing appeal, and overall acceptability. 'Eating quality' sensory analysis descriptors examined were: colour, texture, odour, oxidation flavour 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).

Salmon fillets were cooked for sensory analysis in a Zanussi oven at 180ºC for 12 min until an internal meat temperature of 72ºC was reached. Following cooking, fillets were cooled and the fish was removed from the skin and salmon portions were identified with random three-digit codes. Sample presentation order was randomised to prevent any flavour carryover effects (Macfie et al., 1989). Sensory analysis was undertaken in the panel booths at the university sensory laboratory in accordance with the ISO (2007) International Standard Guidelines. Assessors were also provided with water and crackers to cleanse their palate between samples. Results for sensory analysis scores were measured in centimetres (cm). Results were presented as significance of regression coefficients.

2.2.10 Statistical analysis

All analyses were performed in duplicate. Mean sample values (n = 3) for each of the five treatment groups (PC, 0, 5, 10 and 15% UR) were subjected to statistical analysis. A full repeated measures two-way analysis of variance (ANOVA) was conducted to investigate the effects of dietary *U. rigida* level, time and their interactions. Dietary *U. rigida* level represented the 'between-subjects' factor and the effect of time was measured using the 'within-subjects' factor. Tukey's test was used to adjust for multiple comparisons between treatment means. The analysis was carried out using SPSS 18.0 for Windows (SPSS, Chicago, IL, USA) software package.

'Visual' and 'eating quality' sensory data was analysed with ANOVA-Partial Least Squares Regression (APLSR) to process the mean data accumulated from the 26 test subjects in duplicate. 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. In these models assessor and session level effects were removed using level correction. The validated model explained variance was 13.93% and 11.61% and the calibrated variance was 17.53% and 15.31% on days 1 and 7 respectively. 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). All analyses were performed using the Unscrambler Software, version 9.8 (CAMO ASA, Trondheim, Norway).

2.3 RESULTS AND DISCUSSION

2.3.1 Proximate analysis and pH of salmon fillets

The protein content of fresh (raw) salmon fillets fed with *iso*-nitrogenous diets ranged from ~20 to 21% for all dietary treatments (Table 2.2). Results are in agreement with previously reported protein values (~21%) for farmed Atlantic salmon fillets (Aksnes et al., 1986). Fat levels were unaffected by *U. rigida* supplementation and ranged from ~3 to 4%. Similarly, Valente et al., (2006) reported that the lipid content of European sea bass (*Dicentrarchus labrax*) was not affected by dietary addition of *U. rigida* (0-10%). Moisture and ash levels ranged from ~73 to 75% and ~0.9 to 1.3%, respectively, with no significant differences between dietary treatments. Dietary supplementation of *Ulva spp.* (50:50 *U. rigida* and *U. lactuca*) at 0-20% did not influence moisture and ash levels in Nile tilapia (*Oreochromis niloticus*) (Marinho et al., 2013). In general, the supplementation of Ulva in fish diets does not influence the resulting proximate composition of the fish fillets (Marinho et al., 2013; Valente et al., 2006). In the present study, the composition of salmon fillets from salmon fed *U. rigida* is equivalent to those fed the positive control (PC) diet. Therefore, *U. rigida* is a potentially acceptable replacement ingredient for fish meal in Atlantic salmon feed.

Table 2.2. Effect of dietary *Ulva rigida* (UR) on the proximate composition of fresh salmon fillets.

Treatment	% on wet weight basis			
	Protein	**Moisture**	**Fat**	**Ash**
PC	20.25 ± 1.04[a]	74.89 ± 2.09[a]	3.92 ± 0.19[a]	0.94 ± 0.19[a]
0% UR	20.51 ± 0.49[a]	74.71 ± 0.70[a]	3.89 ± 0.11[a]	0.90 ± 0.11[a]
5% UR	20.55 ± 0.43[a]	73.71 ± 1.24[a]	4.42 ± 1.56[a]	1.32 ± 0.08[a]
10% UR	21.14 ± 0.98[a]	73.93 ± 0.93[a]	3.65 ± 1.93[a]	1.29 ± 0.09[a]
15% UR	20.48 ± 0.81[a]	75.04 ± 0.60[a]	3.47 ± 0.52[a]	1.01 ± 0.43[a]

[a]Within each composition type, mean values (± standard deviation) in the same column are not significantly different, $p > 0.05$.

The pH of the fresh salmon fillets ranged from ~6.14 to 6.25 over the 15 day storage period and was unaffected by the addition of dietary *U. rigida*. The pH values reported are comparable to previously reported values (6.1 – 6.4) for farmed Atlantic salmon during storage (Aksnes et al., 1986).

2.3.2 Colour stability of fresh salmon fillets

The surface lightness 'L*' values significantly increased ($p < 0.05$) in fresh salmon fillets from day 1 to 15 (Table 2.3). Lightness values of the PC and 15% UR groups were significantly lower ($p < 0.05$) compared to 0% UR on day 12 of storage. The lower lightness 'L*' values of PC fillets was attributed to the concurrent increase in a pink flesh pigment concentration provided by deposition of synthetic carotenoid (astaxanthin) in the fish muscle. Similarly, deposition of colour pigments from *U. rigida* in the salmon muscle is most likely responsible for the significantly lower lightness values in 15% UR fillets. This is in agreement with previously reported findings where dietary astaxanthin or canthaxanthin (50 mg/kg feed) increased carotenoid content and simultaneously decreased lightness of Atlantic salmon fillets (Skrede and Storebakken, 1986).

Trends indicated an increase in surface redness 'a*' values of PC salmon fillets over time, however results were not statistically significant ($p > 0.05$) (Table 2.3). Synthetic astaxanthin deposited in the PC salmon muscle resulted in a final red/pink fillet colour. Dietary *U. rigida* (0-15% UR), increased the surface '-a*' greenness of salmon fillets as a function of *U. rigida* concentration which indicated deposition of *U. rigida* colour pigments such as chlorophyll and xanthophylls (lutein and zeaxanthin) in the muscle. Similarly, astaxanthin esters provided red coloration to red sea bream (*Chrysophrys major*) while other carotenoids such as β-carotene, zeaxanthin, lutein

Table 2.3. Effect of dietary *Ulva rigida* (UR) on the surface lightness ('*L**' value), redness ('*a**' value) and yellowness ('*b**' value) values of fresh salmon fillets stored in modified atmosphere packs (60% N_2 : 40% CO_2) for up to 15 days at 4°C.

Treatment		**Storage time at 4°C, days**				
	Parameter	**1**	**3**	**7**	**12**	**15**
PC	Lightness	47.40 ± 2.01^{aA}	48.08 ± 1.89^{aAB}	51.89 ± 1.65^{Aabc}	53.51 ± 2.03^{aBC}	56.99 ± 2.62^{aC}
0% UR	*L**	51.52 ± 2.26^{aA}	51.18 ± 1.58^{aA}	56.29 ± 1.29^{aB}	60.90 ± 1.07^{bC}	60.13 ± 0.51^{aBC}
5% UR		51.00 ± 2.93^{aAB}	50.65 ± 2.48^{aA}	52.82 ± 4.05^{aAB}	58.01 ± 1.54^{abBC}	59.89 ± 1.02^{aC}
10% UR		50.34 ± 0.48^{aA}	49.72 ± 1.76^{aA}	52.99 ± 1.41^{aAB}	56.29 ± 2.60^{abB}	57.26 ± 2.65^{aB}
15% UR		47.23 ± 3.66^{aA}	47.58 ± 0.73^{aA}	52.54 ± 2.42^{aAB}	53.95 ± 3.18^{Aab}	57.16 ± 2.35^{aB}
PC	Redness	3.19 ± 0.29^{aA}	3.68 ± 0.90^{aA}	5.53 ± 1.39^{aA}	4.40 ± 0.63^{aA}	5.17 ± 1.28^{aA}
0% UR	*+a**	-0.90 ± 1.15^{bA}	-1.85 ± 0.22^{bA}	-1.88 ± 0.58^{bA}	-1.47 ± 0.56^{beA}	-1.67 ± 0.10^{bA}
5% UR	Greenness	-3.17 ± 0.75^{cA}	-2.86 ± 0.95^{bA}	-2.29 ± 0.59^{bA}	-3.30 ± 0.91^{bdA}	-3.35 ± 0.38^{bcA}
10% UR	*-a**	-3.64 ± 0.68^{cA}	-3.46 ± 1.97^{bA}	-3.21 ± 0.78^{bA}	-3.47 ± 1.25^{cdeA}	-3.89 ± 1.17^{cA}
15% UR		-4.00 ± 0.72^{cA}	-3.80 ± 0.65^{bA}	-3.26 ± 0.11^{bA}	-3.79 ± 0.51^{cdA}	-4.04 ± 0.21^{cA}
PC	Yellowness	11.70 ± 1.05^{aAB}	11.53 ± 1.75^{aA}	15.02 ± 0.90^{aAB}	13.75 ± 0.49^{aAB}	15.23 ± 1.99^{aB}
0% UR	*b**	4.18 ± 2.50^{bA}	4.29 ± 0.60^{bA}	5.26 ± 1.49^{bA}	6.07 ± 0.45^{bA}	6.37 ± 1.07^{bA}
5% UR		14.83 ± 2.16^{acA}	13.30 ± 2.24^{aA}	13.05 ± 2.41^{aA}	13.55 ± 0.78^{aA}	17.75 ± 2.20^{aA}
10% UR		20.58 ± 4.31^{cdA}	19.04 ± 2.13^{cA}	20.69 ± 1.76^{cA}	21.69 ± 4.30^{cA}	23.15 ± 2.94^{cA}
15% UR		22.54 ± 3.08^{dA}	19.16 ± 1.78^{cA}	22.79 ± 2.82^{cA}	21.89 ± 1.36^{cA}	23.13 ± 1.21^{cA}

^{abcd}Within each parameter and storage day, mean values (± standard deviation) in the same column bearing different superscripts are significantly different, $p < 0.05$.

^{ABC}Within each treatment, mean values (± standard deviation) in the same row bearing different superscripts are significantly different, $p < 0.05$.

and canthaxanthin did not enhance surface redness values of the fillets (Nakazoe et al., 1984).

Chlorophyll *a* (primary photosynthetic) and chlorophyll *b* (accessory pigment) give *U. rigida* its characteristic green colour. *U. rigida* contains a range of carotenoids which include yellow xanthophylls such as lutein and zeaxanthin. Lutein and lutein esters may appear greenish-yellow at low levels or orange-red at higher concentrations (Piccaglia et al., 1998). Torrissen et al., (1989) reported salmon lack the ability to convert yellow xanthophylls to canthaxanthin or astaxanthin before deposition in the muscle which would result in orange/red pigmentation of the fillet. In the present study, deposition of chlorophyll pigments may have contributed to the final greenness colour observed in the salmon fillets, while xanthophyll levels (unconverted) may have been too low to enhance surface redness values.

Trends indicated that the surface yellowness values of fillets (0-15% UR) increased over time however results were not statistically significant ($p > 0.05$). Yellowness (5-15% UR) values were significantly ($p < 0.05$) higher than 0% UR on each storage day (Table 2.3). The increase in surface yellowness was attributed to deposition of xanthophylls (lutein and zeaxanthin) from *U. rigida*. On all days the yellowness of PC and 5% UR were similar. Similarly, Soler-Vila et al., (2009) reported stronger orange flesh tones in rainbow trout (*Oncorhynchus mykiss*) due to the deposition of lutein and zeaxanthin from dietary addition of a red seaweed (*Porphyra dioica*). Previously reported instrumental 'b*' values correspond to increased deposition of yellow xanthophylls in red porgy (*Pagrus pagrus*) and Channel catfish (*Ictalurus punctatus*) (Kalinowski et al., 2005; Li et al., 2007). Yellowness values for 10 and 15% UR were not significantly different on all days indicating deposition of carotenoids in the salmon muscle was limited by Atlantic salmon digestion and metabolism of dietary *U. rigida*.

The capacity of salmon to deposit dietary carotenoids in the muscle develops as the fish grow and is most efficient when fish are about 1kg in size (Shahidi and Brown, 1998). The average weight of the salmon at the end of the present feeding study (~ 521.50 g) was less than 1kg therefore; deposition of carotenoids may have been limited by fish maturity.

Carotenoid pigmentation in salmon flesh is affected by dietary pigment source, level and duration of feeding as well as the degree of carotenoid esterification and structure (Torrissen et al., 1989). Some fish species, such as koi and various crustaceans (*Penaeus japonicus* and *P. monodon*), possess the enzymatic mechanisms required to convert yellow carotenoids into other forms such as red astaxanthin (Lorenz and Cysewski, 2000). Poor deposition of dietary carotenoids in muscle tissues of Atlantic salmon compared to related fish such as rainbow trout and coho salmon is well documented. Approximately 10-15% of dietary astaxanthin is taken up from the intestine and deposited in its unesterified form in Atlantic salmon muscle (Bjerkeng et al., 1999). In the present study, Atlantic salmon were unable to modify the structural properties of yellow dietary carotenoids before deposition in the muscle thus pigments from *U. rigida* were ineffective at providing red coloration to Atlantic salmon fillets. However, due to final yellow/orange flesh colour of salmon fillets, lower levels (5%) of *U. rigida* may be considered as a potential natural alternative yellow/orange pigment source to replace or reduce artificial orange colour sources (i.e. canthaxanthin) currently used in farmed salmon feed.

2.3.3 Lipid oxidation in fresh and cooked salmon fillets and fresh salmon fillet homogenates

In fresh salmon fillets in MAP, levels of lipid oxidation were low with mean values ranging from 0.07-0.14 mg MDA/kg salmon (Table 2.4). The gaseous

Table 2.4. Effect of dietary *Ulva rigida* (UR) on lipid oxidation (TBARS*) in fresh salmon fillets stored in modified atmosphere packs (60% N_2 : 40% CO_2) for up to 15 days at 4°C.

Treatment	**Storage time at 4°C, days**				
	1	**3**	**7**	**12**	**15**
PC	0.076 ± 0.01^{aA}	0.096 ± 0.02^{aA}	0.081 ± 0.03^{aA}	0.100 ± 0.02^{aA}	0.096 ± 0.01^{aA}
0% UR	0.091 ± 0.02^{aA}	0.108 ± 0.02^{aA}	0.074 ± 0.00^{aA}	0.119 ± 0.04^{aA}	0.105 ± 0.02^{aA}
5% UR	0.080 ± 0.02^{aA}	0.120 ± 0.02^{aA}	0.078 ± 0.02^{aA}	0.103 ± 0.03^{aA}	0.103 ± 0.03^{aA}
10% UR	0.066 ± 0.01^{aA}	0.099 ± 0.00^{aAB}	0.072 ± 0.02^{aA}	0.100 ± 0.00^{aAB}	0.137 ± 0.04^{aB}
15% UR	0.070 ± 0.02^{aA}	0.103 ± 0.04^{aA}	0.082 ± 0.02^{aA}	0.099 ± 0.01^{aA}	0.121 ± 0.01^{aA}

[a]Within each storage day, mean values (± standard deviation) in the same column are not significantly different, $p > 0.05$.
[ABC]Within each treatment, mean values (± standard deviation) in the same row bearing different superscripts are significantly different, $p < 0.05$.
*TBARS, mg malondialdehyde (MDA)/kg salmon.

environment within the MAP (60% N_2 : 40% O_2) and the physical condition of the salmon fillets (intact) may have influenced the low levels of lipid oxidation. Similarly, low lipid oxidation values (< 0.75 mg MDA/kg) were reported for salmon fillets stored in MAP (60% CO_2 : 40% N_2) at 3°C (Sivertsvik et al., 1999). Lipid oxidation significantly ($p < 0.05$) increased over the 5 day storage period in cooked salmon fillets (Figure 2.1) and levels of lipid oxidation were higher than in fresh salmon fillets with mean values ranging from 0.06-1.95 mg MDA/kg salmon. The addition of *U. rigida* in salmon diets did not significantly enhance lipid stability in fresh or cooked salmon fillets.

Higher '-a*' (greenness) and 'b*' (yellowness) values, due to the addition of *U. rigida* to salmon diets indicated deposition of colour pigments in muscle tissue. Lutein and zeaxanthin (xanthophylls in *Ulva spp.*) are potent antioxidants in *in vitro* liposomal membrane models (Sujak et al., 1999). Additionally, the antioxidant activity of extracts from *Ulva spp.* (*Ulva clathrata*, *U. prolifera* and *Enteromorpha prolifera*) has been reported using a number of *in vitro* antioxidant assays (DPPH, ABTS, hydroxyl radical scavenging, reducing activity, transition metal ion chelation) (Cho et al., 2011; Farasat et al., 2013a, b). Antioxidant activity of polysaccharides extracted from *Ulva spp.* is strongly linked to its degree of sulphation, molecular weight, chemical composition and chain conformation (Alves et al., 2013). In the present study, despite the antioxidant potential of *U. rigida* no increase in lipid stability of the salmon fillet of UR treatments (0-15%) was observed.

Raw salmon fillet homogenates were subjected to iron ($FeCl_3$) ascorbate-induced lipid oxidation in order to more closely examine the antioxidant potential of *U. rigida* compounds deposited in salmon muscle tissue. Following $FeCl_3$ and ascorbic acid addition, lipid oxidation significantly ($p < 0.05$) increased in all tissue homogenates over the 24 hr storage period. No significant differences were observed between PC and all

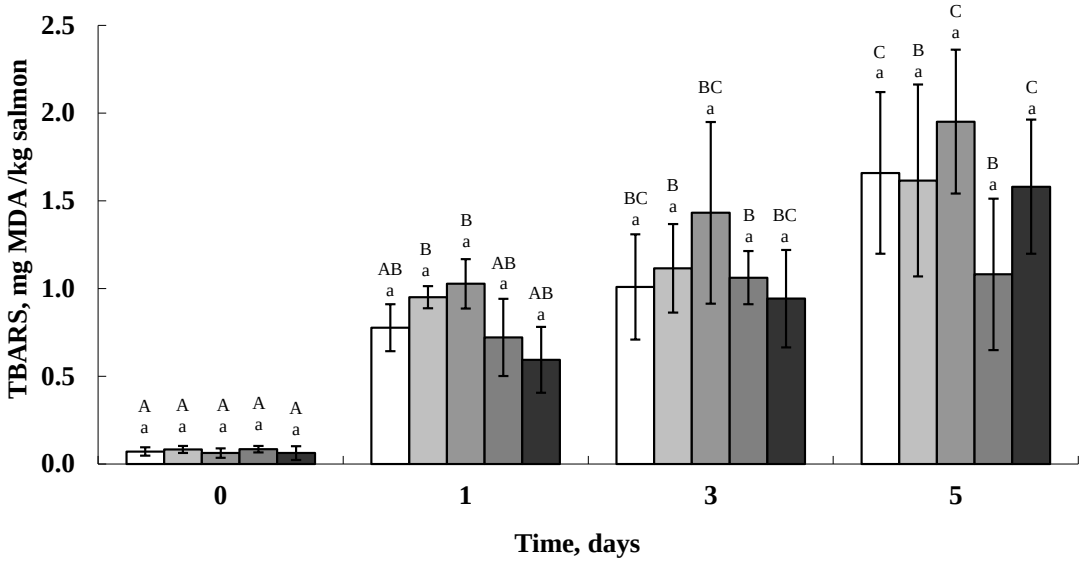

Figure 2.1. Effect of dietary *Ulva rigida* (UR) on lipid oxidation (TBARS) in cooked salmon fillets stored aerobically for up to 5 days at 4°C. [a]Within each storage day, mean values (± standard deviation) are not significantly different, $p > 0.05$. [ABC]Within each treatment, mean values (± standard deviation) bearing different superscripts are significantly different, $p < 0.05$. (□), PC; (▢), 0% UR; (▢), 5% UR; (▢), 10% UR; (■), 15% UR.

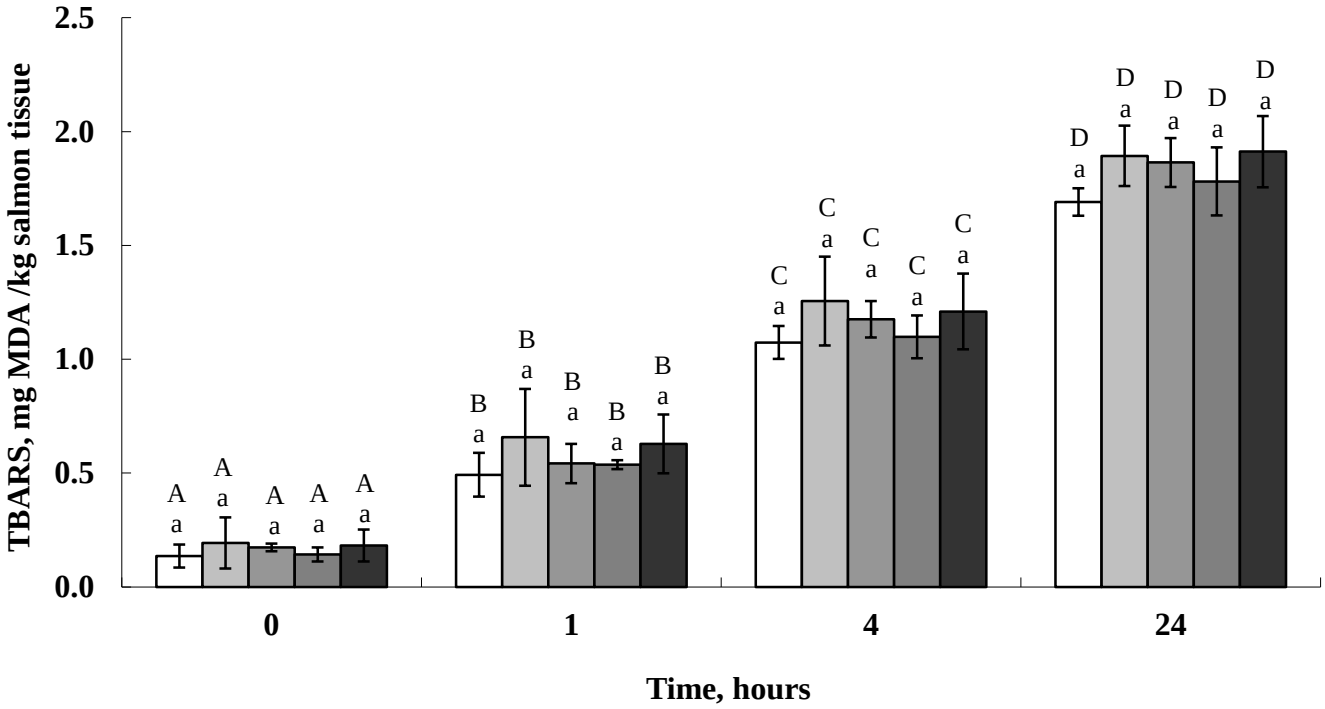

Figure 2.2. Effect of dietary *Ulva rigida* (UR) on lipid oxidation (TBARS) in salmon fillet homogenates containing equimolar $FeCl_3$/ascorbate (45 μM) and stored for 24 hours at 4°C. [a]Within each storage time, mean values (± standard deviation) are not significantly different, $p > 0.05$. [ABCD]Within each treatment, mean values (± standard deviation) bearing different superscripts are significantly different, $p < 0.05$. (□), PC; (□), 0% UR; (□), 5% UR; (□), 10% UR; (■), 15% UR.

UR treatments (0-15%) with mean values ranging from 0.09-2.09 mg MDA/kg salmon (Figure 2.2). Hamre et al. (2004) reported dietary vitamin E was the only agent that actively protected Atlantic salmon fillets against lipid oxidation, while other dietary antioxidants such as vitamin C and astaxanthin (10 and 50 mg/kg feed) had no effect on lipid stability. Torstensen et al. (2001) reported no effects of astaxanthin, vitamin C or vitamin E as antioxidants in Atlantic salmon fillets. In contrast, other fish species such as red porgy (*Pagrus pagrus*) fed synthetic or natural astaxanthin (25 or 50 mg/kg feed) resulted in lower levels of lipid oxidation in the skin of the fish due to deposition of esterified astaxanthin. Terjera et al. (2007) concluded the deposition of different forms of carotenoids between fish species (i.e. porgy vs salmon) is attributed to differences in lipolytic enzyme selectivity towards fatty acids and the rate of hydrolysis may serve to explain differences in substrate efficiency and subsequent uptake rates from the gastrointestinal tract of the fish. Previously reported studies, indicated carotenoids may be used as potential antioxidants in salmon species. However, fish species and products (i.e. salmon vs. rainbow trout steaks), degree of processing and level of carotenoids present in muscle tissues determine if lipid stability can be enhanced (Decker et al., 2000).

In each test system examined (fresh fillets stored in MAP, cooked fillets stored aerobically, and fresh fillet homogenates subjected to iron-ascorbate induced oxidation) levels of lipid oxidation in salmon fillets were unaffected by dietary *U. rigida*. Atlantic salmon possess effective antioxidant defence systems due in part to the large quantities of polyunsaturated fatty acids in muscle tissues and dietary vitamin E is the key antioxidant for protection of salmon fillets during storage. Furthermore, Atlantic salmon reared under normal conditions only respond to increased oxidative stress when other antioxidant defence systems such as glutathione peroxidise and vitamin E are limited

(Torstensen et al., 2001). Martínez-Álvarez et al., (2005) reported younger fish demonstrated higher levels of catalase (CAT) and super oxide dismutase (SOD) antioxidant enzymes along with lower levels of lipid oxidation in liver tissues compared to older fish. In the present study, in addition to dietary *U. rigida*, salmon from each treatment were fed supplementary vitamin A and E (Table 2.1) and slaughtered after 29 weeks. Therefore, the antioxidant potential of *U. rigida* constituents to further enhance the lipid stability of the salmon fillet may have been negated by a stable antioxidant defence system and the age of the salmon. Retention of pigments including carotenoids from *U. rigida* in fish muscle did not offer an increase in lipid stability to the salmon fillets under the conditions tested potentially due to various factors such as the level and chemical composition of *U. rigida* constituents deposited in the salmon fillets, as well as age and antioxidant defence systems of the fish.

2.3.4 Microbiology of fresh salmon fillets

Mesophilic and psychrotrophic total viable counts (TVC) significantly ($p < 0.05$) increased during storage in MAP fresh salmon fillets, and ranged from ~ 0.7 to 7.4 $\log_{10}$ cfu/g and ~ 0.9 to 6.6 $\log_{10}$ cfu/g, respectively (Table 2.5). Similarly, Rasmussen et al. (2002) reported initial colony counts from 2 to 5 $\log_{10}$ cfu/g on day 0 and end of shelf-life determined as colony counts > 7 $\log_{10}$ cfu/g for fresh salmon fillets stored at 4°C. Trends indicated lower mesophilic and psychrotrophic TVC for 5% UR on all days compared to the control, however results were not statistically significant ($p > 0.05$). On day 12, 5% UR psychrotrophic TVC were lower than all treatments ($p < 0.05$).

Limited studies on *Ulva spp.* antimicrobial activity exist, however investigation of green seaweed extracts demonstrated antimicrobial activity is linked to numerous compounds including phenols, fatty acids, indoles, acetogenins, and terpenes

Table 2.5. Effect of dietary *Ulva rigida* (UR) on the microbial status* (mesophilic and psychrotrophic) of fresh salmon fillets stored in modified atmosphere packs (60% N_2 : 40% CO_2) for up to 15 days at 4°C.

Treatment		**Storage time at 4°C, days**				
	Temp	**1**	**3**	**7**	**12**	**15**
PC	30°C	1.13 ± 0.98^{aA}	1.80 ± 0.17^{aA}	5.72 ± 0.16^{aB}	5.71 ± 1.28^{aB}	7.27 ± 0.66^{aB}
0% UR	(mesophilic	1.23 ± 1.08^{aA}	2.12 ± 0.10^{aA}	5.80 ± 0.08^{aB}	6.32 ± 0.46^{aBC}	7.37 ± 0.26^{aC}
5% UR	count)	0.67 ± 1.15^{aA}	2.00 ± 0.30^{aA}	4.67 ± 0.40^{aB}	5.63 ± 0.85^{aB}	6.26 ± 0.35^{aB}
10% UR		1.13 ± 0.98^{aA}	1.96 ± 0.45^{aA}	5.57 ± 0.23^{aB}	5.64 ± 0.66^{aB}	6.48 ± 0.33^{aB}
15% UR		1.23 ± 1.08^{aA}	1.96 ± 0.24^{aA}	5.67 ± 0.05^{aB}	5.74 ± 1.25^{aB}	5.77 ± 0.61^{aB}
PC	4°C	2.70 ± 0.00^{aA}	3.12 ± 0.10^{aA}	5.07 ± 0.51^{aB}	5.45 ± 0.10^{aB}	6.14 ± 0.75^{aB}
0% UR	(psychrotrophic	3.13 ± 0.23^{aA}	3.33 ± 0.84^{aA}	4.64 ± 0.50^{aB}	5.26 ± 0.30^{aB}	6.56 ± 0.24^{aC}
5% UR	count)	1.00 ± 1.73^{aA}	1.96 ± 1.71^{aAB}	3.96 ± 0.24^{aABC}	4.54 ± 0.40^{bBC}	5.90 ± 0.79^{aC}
10% UR		1.00 ± 1.73^{aA}	3.22 ± 0.24^{aAB}	4.28 ± 1.27^{aBC}	5.70 ± 0.20^{aBC}	6.36 ± 0.48^{aC}
15% UR		0.90 ± 1.56^{aA}	2.80 ± 0.17^{aAB}	4.32 ± 0.33^{aBC}	5.27 ± 0.18^{aC}	5.73 ± 0.29^{aC}

[ab]Within each parameter and storage day, mean values (± standard deviation) in the same column bearing different superscripts are significantly different, $p < 0.05$.

[ABC]Within each treatment, mean values (± standard deviation) in the same row bearing different superscripts are significantly different, $p < 0.05$.

*$\log_{10}$CFU (colony forming units)/g salmon

(Parsaeimehr & Chen, 2013). Season of harvest, geographical location, purity, concentration, solvent use and extraction method can influence the antimicrobial activity of seaweed extracts (Hornsey & Hide 1976). El Baky et al., (2009) reported the antibacterial activity of crude extracts of *U. lactuca* against 6 bacterial strains. Antibacterial activity of an *U. rigida* extract was reported as the most effective at inhibition of *Staphylococcus aureus* compared to other brown and red seaweed extracts (Taskin et al., 2007). In a previously reported study, a *Chondrus crispus* extract enhanced the growth of the food spoilage and food pathogenic bacteria up to 66.08% due the presence of certain sugars and proteins in the seaweed which supported bacterial growth (Cox et al., 2010). In the present study, the lack of significant antimicrobial activity in the higher levels of inclusion may have been due to the presence of other compounds in the seaweed as reported by Cox et al. (2010). Dietary *U. rigida* at low levels (5%) resulted in salmon fillets with enhanced microbial stability. Further research is necessary to isolate compounds from *U. rigida* with antimicrobial activity to enhance the shelf-life of salmon fillets.

2.3.5 Sensory evaluation of fresh and cooked salmon fillets

In 'visual' sensory analysis, fillets from fresh salmon fed astaxanthin (PC) were very highly significantly positively correlated to pinkness, purchasing appeal and overall acceptability ($p < 0.001$) on days 1 and 7 of storage (Table 2.6). On days 1 and 7 of storage, salmon fed 10 and 15% UR were significantly negatively correlated with pinkness, purchasing appeal and overall acceptability. The observations of the panellists was in agreement with instrumental '*a**' redness values, where PC had the largest '*a**' values compared to all UR treatments. Purchasing appeal and overall acceptability of 0

Table 2.6. Significance of regression coefficients (ANOVA values) of sensory analysis scores for fresh and cooked salmon fillets as derived by jack-knife uncertainty testing.

Parameter	**Storage time, days / Treatment**									
	PC		**0% UR**		**5% UR**		**10% UR**		**15% UR**	
Visual Sensory Analysis	**1**	**7**	**1**	**7**	**1**	**7**	**1**	**7**	**1**	**7**
Pinkness	0.00***	0.00***	-0.00***	-0.05*	-0.008**	-0.09	-0.01*	-0.008**	-0.008**	-0.00***
Whiteness	-0.00***	-0.00***	0.00***	0.00***	0.007**	0.04*	0.00***	0.006**	0.00***	0.003**
Drip	0.20	0.32	-0.00***	-0.11	-0.05	-0.20	-0.05	-0.36	-0.06	-0.56
Purchasing appeal	0.00***	0.00***	-0.25	-0.71	-0.67	-0.55	-0.001**	-0.002**	-0.00***	-0.002**
Overall acceptability	0.00***	0.00***	-0.09	-0.32	-0.85	-0.80	-0.001**	-0.001**	-0.00***	-0.001**
Eating Quality Sensory Analysis										
Colour	0.00***	0.00***	-0.27	-0.76	-0.48	-0.43	-0.00***	-0.003**	-0.00***	-0.00***
Texture	0.48	-0.61	-0.21	0.83	-0.51	0.99	-0.05	0.60	-0.10	0.56
Odour	-0.47	-0.10	0.77	0.64	0.64	0.80	0.86	0.09	0.83	0.08
Oxidation flavour	-0.27	-0.16	0.99	0.87	0.66	0.49	0.37	0.25	0.39	0.16
Overall acceptability	0.002**	0.003**	-0.16	-0.61	-0.94	-0.30	-0.001	-0.16	-0.002**	-0.06

Significance of regression coefficients; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

and 5% UR fillets were similar indicating sensory panellists were unable to distinguish between fillets with 0 and 5% *U. rigida*. Proximate composition indicated moisture (~74 to 75%) was similar across all treatments (Table 2.2) and dietary *U. rigida* did not influence drip loss on days 1 and 7 of storage. Sivertsvik et al. (1999) reported drip loss was low (~ 0.3%) in salmon stored in MAP (60% CO_2 : 40% N_2).

In 'eating quality' sensory analysis, PC was very highly significantly positively correlated to colour of cooked salmon fillets while 10 and 15% UR was very highly significantly negatively correlated to fillet colour ($p < 0.001$) on both analysis days. After cooking, salmon fillets containing UR appeared whiter than the fresh salmon fillets (5-15% UR). Based on the colour of the cooked salmon fillets, sensory panellists were able to distinguish between the positive control and salmon fed *U. rigida*. Texture, odour and oxidation flavour in cooked salmon fillets were not significantly influenced by dietary UR. The ability of panellists to detect no oxidation/off flavours in the salmon fillets is in agreement with low levels of lipid oxidation (TBARS) reported (Table 2.4). On days 1 and 7, salmon fed PC were significantly positively correlated ($p < 0.01$) to overall acceptability (Table 2.6). On day 1, 15% UR was significantly negatively correlated to overall acceptability ($p < 0.01$). In a previously reported study, based on colour of the fillet, sensory panellists discriminated between rainbow trout (*Oncorhynchus mykiss*) fed a mixture of plant protein sources (corn gluten meal, wheat gluten, extruded peas and rapeseed meal) and the control. Consequently, other organoleptic descriptors (such as hardness, sweetness and odour) were negatively influenced (Lu et al., 2003). In the present study, no significant difference between 0 and 5% UR and overall acceptability was observed, indicating similar preferences by naïve assessors despite their ability to distinguish between fillets based on colour. Therefore, *U. rigida* may be incorporated in

the salmon feed at low levels (5%) with out negatively impacting on texture, odour, oxidation flavours or overall acceptability sensory descriptors.

2.4 CONCLUSIONS

Quality parameters of salmon fillets were not influenced by dietary supplementation of UR due to various factors including the form or concentration of seaweed constituents deposited in the muscle as well as the age, digestion and metabolic factors associated with Atlantic salmon. 5% UR enhanced the microbial status of salmon fillets compared to the controls on day 12. Research is necessary to investigate if purified or refined *U. rigida* extracts can further enhance antimicrobial activity and/or increase lipid stability. Proximate composition of salmon fillets (5-15% UR) was comparable to salmon fed synthetic astaxanthin (PC). Lipid stability and sensory properties of 5% UR were similar to the control (0% UR). Therefore *U. rigida* at lower levels (5%) may be considered as an ingredient for farmed Atlantic salmon feed without negatively impacting fillet quality parameters (proximate composition, lipid stability and sensorial properties). Due to deposition of *U. rigida* pigments (such as lutein and zeaxanthin) salmon fillet surface colour was enhanced with a yellow/orange colour. Optimal dietary inclusion level of *U. rigida* in Atlantic salmon feed was at 5% where surface yellowness 'b*' values of fillets was equivalent to fish fed synthetic astaxanthin. Consumers associate salmon fillets with a typical red/pink colour. Further studies are necessary to investigate the use of red seaweed species with different pigments and carotenoid profiles to potentially colour salmon fillets similar to wild salmon species.

CHAPTER 3

Inclusion of *Palmaria palmata* (red seaweed) in Atlantic salmon diets: Effects on the quality, shelf-life parameters and sensory properties of fresh and cooked salmon fillets

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ABSTRACT

The use of *Palmaria palmata* (PP) as a natural ingredient in farmed Atlantic salmon diets was investigated. The effect of salmon diet supplementation with *P. palmata* (0, 5, 10 and 15%) or synthetic astaxanthin (positive control, PC) for 16 weeks pre-slaughter on quality indices of fresh salmon fillets was examined. Susceptibility of salmon fillets/homogenates to oxidative stress conditions was also measured. In salmon fillets stored in modified atmosphere packs (60% N_2 : 40% CO_2) for up to 15 days at 4ºC, *P. palmata* increased surface '-a*' greenness and 'b*' yellowness values in a dose-dependent manner resulting in a final yellow/orange flesh colour. In general, the dietary addition of *P. palmata* had no effect on pH, lipid oxidation (fresh, cooked and fillet homogenates) and microbiological status. 'Eating quality' sensory descriptors (texture, odour and oxidation flavour) in cooked salmon fillets were not influenced by dietary *P. palmata*. Salmon fed 5% PP were very highly significantly positively correlated ($p < 0.001$) to 'eating quality' overall acceptability. Dietary *P. palmata* was ineffective at providing red coloration in salmon fillets however pigment deposition enhanced fillets with a yellow/orange colour. Carotenoids from *P. palmata* may prove to be a natural pigment alternative to canthaxanthin in salmon feeds.

3.1 INTRODUCTION

The current worldwide production of farmed Atlantic salmon (*Salmo salar*) has increased substantially to over 1,000,000 tonnes with the increased demand for highly nutritious fresh salmon and salmon related products in Japan, the European Union and North America (FAO, 2013). Wild salmon feed on other fish and small crustaceans such as krill containing carotenoid pigments which are responsible for the red-pink salmon colour consumers associate with acceptable salmon fillet quality (Breithaupt, 2007). Since salmon cannot synthesise carotenoids, farmed salmon diets are mostly supplemented with synthetic sources of carotenoids (mainly astaxanthin and to a lesser extent canthaxanthin) in order to pigment farmed salmon flesh similar to wild salmon (Johnston et al., 2006).

In nature, astaxanthin (red) exists as three optical isomers (3R,3'R; 3S,3'S and 3R,3'S), with varying ratios depending on the source. The 3R,3'R is considered most bioavailable and all three isomers are present in different ratios in wild salmon. Synthetic astaxanthin (usually an isomer ratio of 1 : 2 : 1) is absorbed, transported and deposited in salmon flesh similar to natural forms of astaxanthin (Krinsky et al., 2004). Canthaxanthin (orange) is not found in wild Atlantic salmon, but is a minor carotenoid in Pacific salmon species and is used in some farmed salmon feeds in conjunction with astaxanthin (EC Directive 70/524, 2002).

The colour of salmon is regarded as one of the most important quality criterion after freshness (EC Directive 70/524, 2002). Pigmentation from dietary carotenoids (yellow, orange and red) can range from pale faintly pigmented flesh, to strong red tones and is considered a vital aspect of commercial feed formulation and fish management. In addition to flesh pigmentation, carotenoids have been linked to proper growth and survival as well as the possible protection of tissues from oxidative damage (Tacon,

1981). Synthetic pigments traditionally used in salmon feed continue to be utilized with success, however pigments from natural sources should be considered with the increase in consumer preference for natural food additives (Dufossé et al., 2005).

Historically, farmed salmon were fed a diet composed of fish meal (sole protein source) and fish oil (major dietary lipid source). In order to increase sustainability and decrease production costs in aquaculture, substitution of fish meal with alternative protein sources such as soybean, corn gluten and wheat has been investigated previously. Today, many alternative plant protein sources have been successfully incorporated into fish feeds (Bjerkeng et al., 1997; Torstensen et al., 2008). However, to date, limited research has been conducted examining the nutritional value of seaweed (macroalgae) as a potential protein substitute for fish meal replacement. Several sources of biological astaxanthin have been utilised to address consumer demand for natural pigmentation of fish including yeast (*Phaffia rhodozyma*), krill (*Euphausia superba*) or crab wastes, and green micro algae (*Haematococcus pluvialis*) (Breithaupt, 2007).

The average protein content of the red seaweed (Rhodophyta) *Palmaria palmata* (~35%) is comparable to high-protein vegetables such as soybeans, and as such, may be used as an alternative protein source for fish feeds (Mouritsen et al., 2013). Ten amino acids, essential for salmon growth and protein structure, all are present in *P. palmata* (Heen et al., 1993; Morgan et al., 1980). *P. palmata* also contains a variety of fat soluble carotenoids including high levels of lutein (yellow), α and *β*-carotene (reddish yellow) as well as chlorophyll *a*/*b* (mid green to olive green) (Chu, 2012; Dawson, 2007). Carotenoids are isoprenoid molecules which aid in the absorption of sunlight and protect cells from oxidative stress by quenching singlet oxygen (Krinsky et al., 2004). The carotenoid content of *P. palmata* varies seasonally and depends on postharvest treatment and is generally present in higher levels compared to other seaweed species (Morgan et

al., 1980). Red seaweeds differ in structure and photosynthetic pigments to green and brown seaweeds with the red colour attributed to the presence of water soluble light-harvesting pigments known as phycobiliproteins, found in chloroplasts, which mask the other fat-soluble pigments. There are three major classes of phycobiliproteins; phycoerythrin (red), phycocyanin (blue) and allophycocyanin (green-blue) (Sidler, 2004).

Individually, colour pigments (carotenoids, xanthophylls, chlorophyll and phycobiliproteins) found in *P. palmata* demonstrated potent antioxidant activity in a number of test systems including *in vitro* antioxidant assays (DPPH, ABTS) and the inhibition of conjugated dienes and TBARS products in a linoleic acid emulsion (Pangestuti & Kim, 2011; Yuan et al., 2005). Pigments have also been reported to possess a range of health promoting properties. Carotenoids and chlorophyll derivatives have exhibited anticancer properties, while phycobiliproteins have been linked to significant anti-inflammatory, hepatoprotective and free radical scavenging properties (Bendich & Olson, 1989; Khattar & Kaur, 2009; Pangestuti & Kim, 2011; Wijesinghe & Jeon, 2012).

Seaweeds may offer potential for use as a fish meal replacer (protein source), in addition to a source of natural pigments and bioactive compounds in fish diets. Therefore the use of seaweed in salmon diets may satisfy an increased consumer demand for natural, health-promoting products and merits investigation. The objective of the study was to examine the effect of including *P. palmata* (0-15%) in farmed salmon diets, on quality, shelf-life parameters and sensory properties of salmon fillets.

3.2 MATERIALS AND METHODS

3.2.1 Reagents

All chemicals used were 'AnalaR' grade and were obtained from Sigma-aldrich Chemie GmbH, Steinheim, Germany; Oxoid Ltd., Basingstoke, Hampshire, England; Merck KGaA, Darmstadt, Germany. *Palmaria palmata* was harvested from the coasts of Galway bay, Ireland.

3.2.2 Salmon and diets

After harvesting, *P. palmata* was subsequently washed and dried before addition to the salmon diet formulations. Diets were prepared at the feed formulation laboratory in Carna Research Station, Ryan Institute, National University of Ireland, Galway. All diets were formulated to be *iso*-nitrogenous (40%), *iso*-lipidic (25%) and *iso*-caloric (26 MJ kg^{-1}). The composition of the experimental diets is outlined in Table 3.1.

Table 3.1. Composition of the experimental salmon diets with inclusion of *Palmaria palmata* (g kg^{-1}, unless otherwise indicated) (adapted from Wan et al. 2014).

	PC	**0% PP**	**5% PP**	**10% PP**	**15% PP**
Fish meal[a]	407.4	407.4	390.8	374.1	357.5
Fish oil[a]	200.0	200.0	201.4	202.8	204.1
Palmaria palmata	-	-	50.0	100.0	150.0
Corn starch[b]	187.3	187.6	152.8	118.1	83.4
Lysamine[c]	90.0	90.0	90.0	90.0	90.0
Glutalys[c]	90.0	90.0	90.0	90.0	90.0
Mineral & vitamin premix[d]	20.0	20.0	20.0	20.0	20.0
Lucantin Pink[e]	0.30	-	-	-	-
Barox Plus[f]	5.00	5.00	5.00	5.00	5.00

[a]United fish products Ltd., Donegal, Ireland.
[b]Laboratory grade, Sigma –Aldrich Company Ltd., Poole, UK.
[c]Purified feed ingredients, Roquette, France.
[d]Premier nutrition products Ltd., Rugeley, UK. (Manufacturers analysis: Ca-12.09%, Ash-78.708%, Na-8.858%, Vitamin A-1.0µg/kg, Vitamin D3 0.10%, Vitamin-E 7.0 g/kg, Cu-250 mg, Mg 15.6 g/kg and P 5.2 g/kg).
[e]BASF, Ludwigshafen, Germany.
[f]Kemin Europa N.V., Herentals, Belgium.

Atlantic salmon smolts (*Salmo salar*) were obtained from a commercial company based at Lough Fee, Connemara, Ireland. After an 8 week acclimation period, a 16-week completely randomised experimental feed trial was carried out at the Carna Research Station. Salmon (n = 33, average fish weight ~ 170.4g) were randomly assigned to one of fifteen tanks (three tanks per treatment) which consisted of five different experimental formulated diets. The positive control group (PC) were fed a basal diet plus synthetic astaxanthin (0.3 g/kg feed) (Table 3.1). The remaining four groups were fed the basal diet plus *P. palmata* (PP) at 0 (0% PP), 5 (5% PP), 10 (10% PP) and 15% (15% PP) inclusion levels. The salmon were hand-fed on five occasions over the course of each day and housed in 1000 L tanks fed by a filtered flow-through seawater supply (ambient temperature regime). At the end of the feeding trial, salmon (average fish weight ~ 419.90 g), were euthanised with a sharp blow to the head followed by the pithing of the brain and were gutted. Fresh (raw) salmon fillets were transported on ice at 4ºC to the School of Food and Nutritional Sciences at University College Cork, Ireland. Further experimental site details, feeding trial conditions and the growth of the fish are reported in Wan et al. 2014.

3.2.3 Proximate analysis of fresh salmon fillets

The proximate composition of fresh salmon fillets was reported on a wet weight basis. Protein (nitrogen x 6.25) was determined by the Kjeldahl method of the Association of Official Analytical Chemists (AOAC, 1995). The moisture and fat content were measured using the SMART Trac rapid moisture/fat analyser (CEM Corporation, NC, USA). The ash content was determined using a muffle furnace (AOAC, 1923). Compositional analysis results were expressed as % on wet weight basis.

3.2.4 Salmon processing and packaging

Fresh salmon fillets (~100 g) (PC, 0, 5, 10 and 15% PP) were placed in low oxygen permeable (<1cm^3/m^2/24 hr at STP) polystyrene/ethylvinylalcohol/polyethylene trays. Trays were covered with a low oxygen permeable (3 cm^3/m^2/24 hr at STP) laminated barrier film with a polyolefin heat-sealable layer. Using modified atmosphere packaging technology, the trays were flushed with 60% N_2 : 40% CO_2 using a vacuum-sealing unit (VS 100, Gustav Müller and Co. KG, Bad Homburg, Germany) equipped with a gas mixer (Witt-Gasetechnik GmbH and Co. KG, Witten, Germany) and heat-sealed. Fresh salmon fillets were stored for up to 15 days under fluorescent lighting conditions (approximately 660 lx) at 4°C. The gas atmosphere (% O_2 and % CO_2) in the modified atmosphere packs (MAP) was checked using a CheckMate 9900 (PBI-DanSensor, Denmark). Immediately after gas flushing, MAP contained 56.91 ± 0.53% N_2 and 42.95 ± 0.42% CO_2. The average gas composition in MAP at the end of the 15 day storage period was 60.40 ± 1.66% N_2 and 39.60 ± 1.66% CO_2. Percent nitrogen was calculated by difference of % O_2 and % CO_2.

In a cooked fish study, salmon fillets from all treatments (PC, 0, 5, 10 and 15% PP) were placed on aluminium foil lined trays and cooked at 180°C for 12 min in a fan-assisted convection oven (Zanussi Professional, Model 10 GN1/1, Conegliano, Italy) until an internal temperature of 72°C was reached. Cooked fillets were placed in trays over-wrapped with oxygen permeable film and stored aerobically for up to 5 days at 4°C.

3.2.5 Measurement of salmon fillet quality and shelf-life parameters

Salmon fillet pH, colour (CIE 'L*' lightness, 'a*' redness and 'b*' yellowness values), lipid oxidation (2-thiobarbituric acid reactive substances (TBARS)), microbiological analysis (mesophilic and psychrotrophic total viable counts) and sensory

evaluation ('visual' and 'eating quality') were measured at intervals during storage as described in Chapter 2 (sections 2.2.5-2.2.9). The pH, colour measurements, lipid oxidation and microbiological analysis of fresh salmon fillets were recorded on days 1, 3, 7, 12 and 15 of storage. In cooked salmon fillets stored aerobically, lipid oxidation was measured on days 0, 1, 3 and 5 of storage. Sensory analysis ('visual' and 'eating quality') of fresh salmon fillets stored in 60% N_2 : 40% CO_2 was performed in duplicate by 26 naïve assessors on days 1 and 7 of storage. 'Visual' sensory analysis descriptors of fresh salmon fillets were pinkness, whiteness, drip, purchasing appeal and overall acceptability. 'Eating quality' sensory analysis descriptors of cooked salmon fillets (180°C for 12 min in a fan-assisted convection oven) were colour, texture, odour, oxidation flavour and overall acceptability.

3.2.6 Statistical analysis

All analyses were performed in duplicate. Mean sample values (n = 3) for each of the five treatment groups (PC, 0, 5, 10 and 15% PP) were subjected to statistical analysis. A full repeated measures two-way analysis of variance (ANOVA) was conducted to investigate the effects of dietary *P. palmata* level, time and their interactions. Dietary *P. palmata* level represented the 'between-subjects' factor and the effect of time was measured using the 'within-subjects' factor. Tukey's test was used to adjust for multiple comparisons between treatment means. The analysis was carried out using SPSS 18.0 for Windows (SPSS, Chicago, IL, USA) software package.

'Visual' and 'eating quality' sensory data was analysed with ANOVA-Partial Least Squares Regression (APLSR) to process the mean data accumulated from the 26 test subjects in duplicate. 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. In these models assessor and session level effects were removed using level correction. The validated model explained variance was -0.51% and 6.27% and the calibrated variance was 2.38% and 8.97% on days 1 and 7 respectively. 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 the Unscrambler Software, version 9.8 (CAMO ASA, Trondheim, Norway).

3.3 RESULTS AND DISCUSSION

3.3.1 Proximate analysis and pH of salmon fillets

The protein contents of fresh (raw) salmon fillets fed with *iso*-nitrogenous diets ranged from ~20 to 22% for all dietary treatments (Table 3.2). Similarly, Bjerkeng et al. (1997) reported a protein content of ~18% for Atlantic salmon fillets from fish fed fish meal or soybean as a fish meal replacer. Levels of fat ranged from ~2 to 3% with no significant differences between dietary treatments. In a similar study, the lipid content of *Oncorhynchus mykiss* (rainbow trout) fillets was not affected by dietary addition of *Porphyra dioica* (red algae) at levels ranging from 0-15% (Soler-Vila et al. 2009). Moisture and ash levels were unaffected by *P. palmata* supplementation with levels ranging from ~74 to 76% and ~1.2 to 1.4%, respectively. Several studies have reported no effect on salmon fillet proximate composition when plant based meals are used as alternative protein and lipid sources compared to traditional feed (fish meal and oil) (Bjerkeng et al., 1997; Rasmussen, 2007). In the present study, the proximate analysis of salmon fillets from salmon fed *P. palmata* is comparable to those fed the positive control (PC) diet. Therefore, *P. palmata* may be used as a potential replacement functional ingredient for fish meal in Atlantic salmon feed without detrimentally affecting the proximate composition of salmon fillets.

Table 3.2. Effect of dietary *Palmaria palmata* (PP) on the proximate composition of fresh salmon fillets.

Treatment	% on wet weight basis			
	Protein	**Moisture**	**Fat**	**Ash**
PC	21.13 ± 0.82[a]	75.45 ± 1.56[a]	2.13 ± 0.89[a]	1.29 ± 0.12[a]
0% PP	21.01 ± 0.61[a]	75.49 ± 1.40[a]	2.27 ± 1.02[a]	1.23 ± 0.19[a]
5% PP	22.00 ± 0.59[a]	73.74 ± 1.90[a]	2.85 ± 1.45[a]	1.40 ± 0.22[a]
10% PP	22.45 ± 1.26[a]	74.40 ± 1.77[a]	1.83 ± 0.74[a]	1.32 ± 0.12[a]
15% PP	20.52 ± 0.66[a]	74.89 ± 2.14[a]	3.32 ± 1.71[a]	1.27 ± 0.12[a]

[a]Within each composition type, mean values (± standard deviation) in the same column are not significantly different, $p > 0.05$.

The pH of the fresh salmon fillets ranged from ~6.27 to 6.33 over the 15 day storage period and was unaffected by the addition of dietary *P. palmata*. Similarly, Einen et al. (2002) reported fillet pH stability (6.3) over storage time for up to 100 hours of Atlantic salmon (*Salmo salar*).

3.3.2 Colour stability of fresh salmon fillets

Lightness values of the PC group were significantly lower ($p < 0.05$) compared to 15% PP on day 1 and to 0 and 10% PP on day 7 of storage (Table 3.3). The lower lightness 'L*' values of PC fillets was attributed to the concurrent increase in flesh pigment concentration provided by deposition of synthetic astaxanthin in the fish muscle. Similarly, Buttle et al. (2001) reported a similar pattern of decreased lightness in fresh salmon fillets from Atlantic salmon fed astaxanthin, canthaxanthin or an astaxanthin/canthaxanthin mix (0.06 g/kg feed).

Trends indicated an increase in surface redness 'a*' values of PC salmon fillets over time, however results were not statistically significant ($p > 0.05$) (Table 3.3). The increased surface redness due to synthetic astaxanthin deposited in the PC salmon fillets resulted in a red/pink colour. In the salmon fed *P. palmata* (0-15% PP), the surface '-a*' greenness of salmon fillets increased as a function of *P. palmata* concentration. The increase in surface greenness as a function of dietary *P. palmata* indicated deposition of pigments which were ineffective at providing red coloration. Similarly, Chatzifotis et al. (2005) reported the inefficacy of the red carotenoid lycopene, contained in tomatoes, in providing red coloration in red porgy (*Pagrus pagrus*). *P. palmata* contains other pigments available for absorption and deposition in the fish muscle such as chlorophyll *a*/*b* (green) and phycobiliproteins which include R-phycocyanin (greenish blue), allophycocyanin

Table 3.3. Effect of dietary *Palmaria palmata* (PP) on the surface lightness ('*L**' value), redness ('*a**' value) and yellowness ('*b**' value) values of fresh salmon fillets stored in modified atmosphere packs (60% N_2 : 40% CO_2) for up to 15 days at 4°C.

Treatment		**Storage time at 4°C, days**				
	Parameter	**1**	**3**	**7**	**12**	**15**
PC	Lightness	41.29 ± 2.06^{aA}	44.72 ± 1.81^{aAB}	45.09 ± 2.13^{bAB}	48.21 ± 1.76^{aB}	48.75 ± 3.86^{aB}
0% PP	*L**	44.73 ± 2.35^{abA}	45.54 ± 2.05^{aAB}	50.42 ± 1.23^{aBC}	51.03 ± 2.05^{aC}	49.67 ± 2.05^{aABC}
5% PP		46.78 ± 2.11^{abA}	45.82 ± 0.50^{aA}	49.78 ± 3.01^{abA}	50.56 ± 0.90^{aA}	49.88 ± 2.09^{aA}
10% PP		46.85 ± 2.79^{abA}	47.44 ± 3.30^{aA}	50.49 ± 0.97^{aA}	49.59 ± 1.36^{aA}	50.02 ± 3.00^{aA}
15% PP		48.24 ± 1.40^{bAB}	45.72 ± 1.00^{aA}	49.28 ± 0.25^{abBC}	49.21 ± 0.62^{aBC}	52.10 ± 1.73^{aC}
PC	Redness	5.63 ± 1.47^{bA}	6.69 ± 1.61^{bA}	6.38 ± 1.79^{bA}	6.08 ± 0.84^{bA}	8.29 ± 1.32^{bA}
0% PP	*+a**	-0.61 ± 0.41^{aA}	-0.53 ± 0.43^{aA}	-0.63 ± 0.27^{aA}	-1.07 ± 0.15^{aA}	-0.48 ± 1.02^{aA}
5% PP	Greenness	-1.21 ± 0.26^{aA}	-1.07 ± 0.68^{aA}	-0.55 ± 0.82^{aA}	-1.11 ± 1.24^{aA}	-1.01 ± 0.15^{aA}
10% PP	*-a**	-1.54 ± 0.28^{aA}	-0.54 ± 1.01^{aA}	-0.71 ± 0.40^{aA}	-1.22 ± 0.64^{aA}	-2.29 ± 0.09^{aA}
15% PP		-1.35 ± 0.59^{aA}	-1.49 ± 0.18^{aA}	-1.27 ± 0.95^{aA}	-1.49 ± 0.89^{aA}	-2.11 ± 0.36^{aA}
PC	Yellowness	10.84 ± 0.92^{bA}	11.50 ± 2.00^{bA}	11.10 ± 0.76^{bA}	12.81 ± 0.73^{bA}	12.81 ± 1.47^{bA}
0% PP	*b**	2.52 ± 0.38^{aA}	2.58 ± 0.47^{aAB}	4.16 ± 1.83^{aAB}	4.05 ± 0.65^{aAB}	4.98 ± 0.16^{aB}
5% PP		3.88 ± 0.46^{aA}	5.05 ± 0.31^{acA}	6.12 ± 1.57^{acA}	6.14 ± 1.43^{acA}	5.59 ± 0.37^{aA}
10% PP		6.54 ± 0.41^{cA}	7.47 ± 0.15^{cdA}	8.60 ± 0.95^{bcA}	8.00 ± 1.69^{cA}	7.92 ± 1.73^{acA}
15% PP		8.98 ± 1.32^{bA}	8.58 ± 0.58^{dA}	10.43 ± 0.37^{bA}	8.96 ± 1.54^{cA}	11.01 ± 2.40^{bcA}

^{ab}Within each parameter and storage day, mean values (± standard deviation) in the same column bearing different superscripts are significantly different, $p < 0.05$.

^{ABC}Within each treatment, mean values (± standard deviation) in the same row bearing different superscripts are significantly different, $p < 0.05$.

(greenish blue), R-phycoerythrin (red) and *β*-phycoerythrin (red) (Prasanna et al., 2007). In the present study, chlorophyll, R-phycocyanin and allophycocyanin, all of which exhibit green colour and may have contributed to the final greenness colour observed in the salmon fillets.

General trends indicated that the surface yellowness 'b*' values of fillets (0-15% PP) increased over time however results were not statistically significant ($p > 0.05$) (Table 3.3). Yellowness (10 and 15% PP) values were significantly ($p < 0.05$) higher than 0% PP on each storage day (Table 3.3). On days 1, 7 and 15 the yellowness of PC and 15% PP were similar. In a previously reported study, Rainbow trout fed diets containing *P. dioica* (0-15%) with no added astaxanthin, exhibited stronger orange flesh tones in fillets as a function of the red algae concentration. It was concluded that red algae contain fat soluble yellow xanthophylls, especially lutein and zeaxanthin which contributed to the final flesh coloration (Soler-Vila et al., 2009). Olsen & Baker (2006) reported the absorption and deposition of lutein, structurally similar to astaxanthin, in Atlantic salmon muscle. In the present study, the increased surface yellowness is most likely due to deposition of fat soluble carotenoids, mainly lutein, from *P. palmata*.

Several factors influence carotenoid deposition in the fish muscle, including quantity available, carotenoid structure and the ability of salmon to metabolise or biotransform available carotenoids. The final carotenoid structure and level of deposition will determine the final colour of the fillet (Breithaupt, 2007). In the present study, the carotenoid profile (lutein, α and *β*-carotene) of *P. palmata* was not sufficient to increase surface redness of the salmon fillets, however the carotenoids did enhance surface yellowness. In recent years, fish feed ingredients include many plant sources like maize gluten with significant amounts of yellow pigments from carotenoids such as lutein and zeaxanthin (Chimsung, 2012). Similarly, canthaxanthin, sometimes used in farmed

salmon feed, imparts an orange colour in salmon flesh, therefore is used in conjunction with astaxanthin to achieve desired final red/pink pigmentation (Buttle, 2001). Due to final yellow/orange flesh colour of salmon fillets, in the present study, the carotenoids from *P. palmata* may be considered as a potential natural pigment alternative to the use of canthaxanthin in farmed salmon feed.

3.3.3 Lipid oxidation in fresh and cooked salmon fillets and fresh salmon fillet homogenates

Overall the levels of lipid oxidation in fresh salmon fillets in MAP were low with mean values ranging from 0.07-0.11 mg MDA kg^{-1} salmon (Table 3.4). The low levels of lipid oxidation may be attributed to physical condition of the salmon fillets (intact) and the gaseous environment within the MAP (60% N_2 : 40% CO_2). Randell et al. (1999) also reported low lipid oxidation values (< 1 mg MDA kg^{-1}) for salmon fillets stored in MAP (60% CO_2 : 40% N_2 or 40% CO_2 : 60% N_2) at 2°C. Processes which change the physical structure of muscle foods such as grinding or mincing and cooking accelerate lipid oxidation reactions. In cooked salmon fillets, lipid oxidation significantly ($p < 0.05$) increased over the 5 day storage period (Figure 3.1) and levels of lipid oxidation were higher than in fresh salmon fillets with mean values ranging from 0.33-1.29 mg MDA kg^{-1} salmon. The addition of *P. palmata* in salmon diets did not significantly influence lipid oxidation in fresh or cooked salmon fillets.

The increase in '-a*' (greenness) and 'b*' (yellowness) colour measurements with increasing dietary *P. palmata* concentration indicated deposition of colour pigments in the muscle. The antioxidant potential *in vitro* of both fat and water soluble compounds (lutein, β-carotene, phycobiliproteins and chlorophyll) of *P. palmata* is well documented (Munir et al., 2013). Additionally, the antioxidant activity of extracts from *P. palmata*

Table 3.4. Effect of dietary *Palmaria palmata* (PP) on lipid oxidation (TBARS) in fresh salmon fillets stored in modified atmosphere packs (60% N_2 : 40% CO_2) for up to 15 days at 4°C.

Treatment	Storage time at 4°C, days				
	1	**3**	**7**	**12**	**15**
PC	0.103 ± 0.02^{aA}	0.073 ± 0.01^{aAB}	0.098 ± 0.00^{aAB}	0.071 ± 0.01^{aB}	0.101 ± 0.01^{aA}
0% PP	0.089 ± 0.01^{aAB}	0.077 ± 0.01^{aAB}	0.101 ± 0.01^{aA}	0.057 ± 0.02^{aB}	0.102 ± 0.00^{aA}
5% PP	0.084 ± 0.02^{aAB}	0.065 ± 0.01^{aA}	0.105 ± 0.01^{aB}	0.070 ± 0.02^{aAB}	0.069 ± 0.01^{aAB}
10% PP	0.088 ± 0.02^{aA}	0.062 ± 0.00^{aA}	0.092 ± 0.01^{aA}	0.066 ± 0.02^{aA}	0.090 ± 0.00^{aA}
15% PP	0.081 ± 0.02^{aA}	0.065 ± 0.01^{aA}	0.089 ± 0.01^{aA}	0.084 ± 0.01^{aA}	0.093 ± 0.01^{aA}

[a]Within each storage day, mean values (± standard deviation) in the same column are not significantly different, $p > 0.05$.
[AB]Within each treatment, mean values (± standard deviation) in the same row bearing different superscripts are significantly different, $p < 0.05$.
*TBARS, mg malondialdehyde (MDA)/kg salmon.

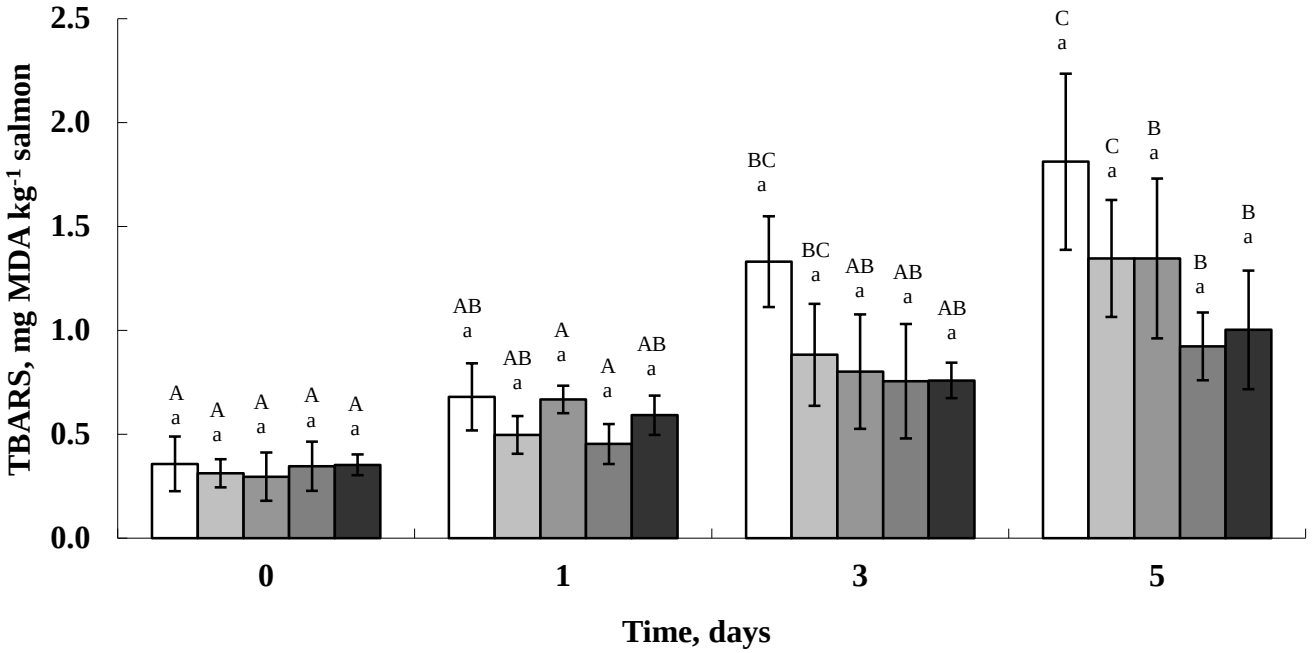

Figure 3.1. Effect of dietary *Palmaria palmata* (PP) on lipid oxidation (TBARS) in cooked salmon fillets stored aerobically for up to 5 days at 4°C. [a]Within each storage day, mean values (± standard deviation) are not significantly different, $p > 0.05$. [ABC]Within each treatment, mean values (± standard deviation) bearing different superscripts are significantly different, $p < 0.05$. (□), PC; (▒), 0% PP; (▒), 5% PP; (▒), 10% PP; (■), 15% PP.

has been reported using a number of *in vitro* antioxidant assays (deoxyribose assay, DPPH, ABTS, TBARS, reducing activity, transition metal ion chelation) (Yuan et al., 2005). Previous studies indicated that dietary antioxidants such as α-tocopherol fed to Atlantic Halibut enhanced lipid stability in fish muscle where significantly lower levels of lipid oxidation was reported in fish fillets (Ruff et al., 2002). Despite the antioxidant potential of *P. palmata* no increase in lipid stability of the salmon fillet was observed in the present study.

To further examine the antioxidant potential of compounds from *P. palmata* deposited in the fish muscle, raw salmon fillet homogenates were subjected to iron ($FeCl_3$) ascorbate-induced lipid oxidation. Following $FeCl_3$ and ascorbic acid addition, lipid oxidation significantly ($p < 0.05$) increased in all tissue homogenates over the 24 hr storage period. No significant differences were observed between PC and all PP treatments (0-15%) with mean values ranging from 0.21-1.77 mg MDA kg^{-1} salmon (Figure 3.2). Similarly, astaxanthin deposited in farmed Atlantic salmon fillets demonstrated no significant antioxidant activity under standardised conditions of accelerated oxidation. Although no effects of astaxanthin as an antioxidant in fish muscle was measured, it was concluded that retention of the carotenoid may offer other beneficial biological effects such as enhanced immune response, inhibition of mutagensis and reduction of photo-induced nuclear damage in cells and tissues (Bendich & Olson, 1989; Ruff et al., 2002).

Under conditions employed in the present study (fresh fillets stored in MAP, cooked fillets stored aerobically, and fresh fillet homogenates subjected to iron-ascorbate induced oxidation) levels of lipid oxidation in salmon fillets were unaffected by dietary *P. palmata*. However, *in vitro* antioxidant activity of *P. palmata* has been reported using

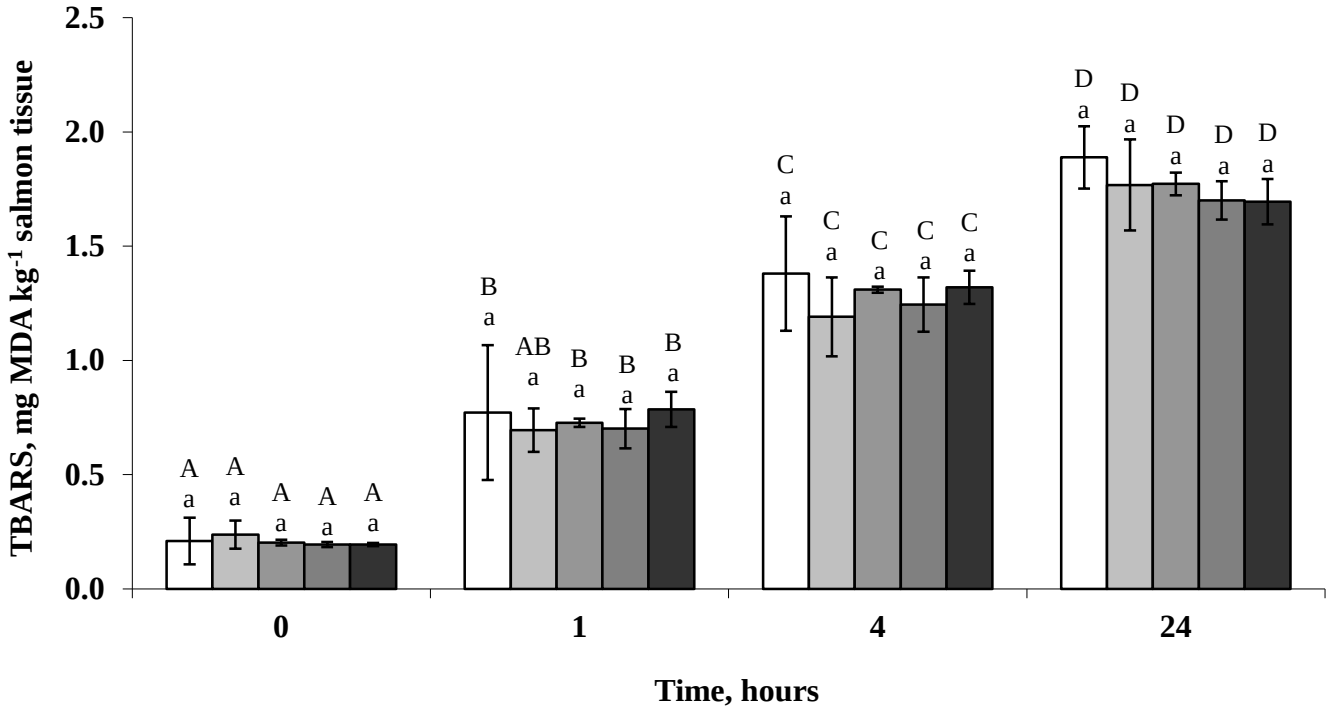

Figure 3.2. Effect of dietary *Palmaria palmata* (PP) on lipid oxidation (TBARS) in salmon fillet homogenates containing equimolar $FeCl_3$/ascorbate (45 μM) and stored for 24 hours at 4°C. [a]Within each storage time, mean values (± standard deviation) are not significantly different, $p > 0.05$. [ABCD]Within each treatment, mean values (± standard deviation) bearing different superscripts are significantly different, $p < 0.05$. (□), PC; (▒), 0% PP; (▒), 5% PP; (▒), 10% PP; (■), 15% PP.

extracts with purified bioactive components and at concentrations greater than those tested in the present study. The antioxidant activity of carotenoids and the other compounds, *in vivo*, depends on numerous factors, such as form, concentration, cellular distribution and interaction with other components present in muscle tissue (Krinsky, 2004). In the present study, the lack of antioxidant activity of pigments in salmon fillets may have been due to form or concentration of components deposited in the muscle.

Although retention of pigments from *P. palmata* did not offer an increase in lipid stability to the salmon fillets under the conditions tested, carotenoids are still necessary for salmon growth and development (Tacon, 1981). Natural pigments retained in salmon fillets, may offer added benefit to consumers. Lutein, for example, has been linked to maintenance of normal visual function in the human eye macula (Christaki et al., 2013). Further studies would need to examine the bioavailability of colour pigments deposited in the fish muscle.

3.3.4 Microbiology of fresh salmon fillets

Mesophilic and psychrotrophic total viable counts (TVC) significantly ($p < 0.05$) increased during storage in MAP in fresh salmon fillets, and ranged from ~1.9 to 9.1 $\log_{10}$ cfu g^{-1} and ~2.3 to ~9.0 $\log_{10}$ cfu g^{-1}, respectively (Table 3.5). Mesophilic counts obtained were similar to previously reported values for fresh salmon fillets stored at 4°C under normal conditions where initial colony counts ranged from 2 to 5 $\log_{10}$ cfu g^{-1} on day 0 and end of shelf-life determined as colony counts > 7 $\log_{10}$ cfu g^{-1} (Rasmussen et al., 2002). In the present study, on day 12 mesophilic and psychrotrophic counts of all salmon fillets were ~ 8 $\log_{10}$ cfu g^{-1}, and considered unsafe for human consumption. The TVC for 5% PP, 10% PP and 15% PP were similar ($p > 0.05$) for all treatments, on each storage day, compared to 0% PP and PC indicating that dietary *P. palmata* did not result

Table 3.5. Effect of dietary *Palmaria palmata* (PP) on the microbial status* (mesophilic and psychrotrophic) of fresh salmon fillets stored in modified atmosphere packs (60% N_2 : 40% CO_2) for up to 15 days at 4°C.

Treatment		**Storage time at 4°C, days**				
	Temp	**1**	**3**	**7**	**12**	**15**
PC	30°C	1.93 ± 0.40^{aA}	2.69 ± 0.64^{aA}	6.83 ± 0.98^{aB}	8.39 ± 0.62^{aC}	9.27 ± 0.59^{aC}
0% PP	(mesophilic	2.03 ± 0.35^{aA}	1.66 ± 1.52^{aA}	5.41 ± 1.00^{aB}	8.38 ± 0.33^{aC}	9.01 ± 1.39^{aC}
5% PP	count)	2.35 ± 0.37^{aA}	2.63 ± 0.49^{aA}	5.98 ± 1.22^{aB}	7.74 ± 1.19^{aBC}	8.87 ± 0.66^{aC}
10% PP		1.78 ± 1.56^{aA}	2.81 ± 0.36^{aA}	5.67 ± 0.76^{aB}	7.37 ± 1.07^{aBC}	9.38 ± 0.27^{aC}
15% PP		1.43 ± 1.25^{aA}	1.49 ± 1.29^{aA}	6.55 ± 0.92^{aB}	8.40 ± 0.46^{aBC}	9.14 ± 0.22^{aC}
PC	4°C	1.96 ± 1.71^{aA}	3.59 ± 1.01^{aA}	6.57 ± 0.75^{aB}	8.61 ± 0.54^{aB}	8.89 ± 0.56^{aB}
0% PP	(psychrotrophic	1.90 ± 1.65^{aA}	3.26 ± 0.73^{aAB}	5.35 ± 0.96^{aB}	8.55 ± 0.28^{aC}	9.44 ± 1.30^{aC}
5% PP	count)	1.13 ± 1.96^{aA}	2.37 ± 2.12^{aAB}	5.86 ± 0.90^{aBC}	7.54 ± 1.34^{aC}	8.69 ± 0.91^{aC}
10% PP		3.43 ± 0.43^{aA}	2.28 ± 2.10^{aA}	5.56 ± 0.88^{aBC}	7.70 ± 0.70^{aCD}	8.89 ± 0.12^{aD}
15% PP		3.18 ± 0.31^{aA}	2.48 ± 2.15^{aA}	6.60 ± 1.03^{aB}	8.59 ± 0.48^{aB}	9.18 ± 0.22^{aB}

[a]Within each parameter and storage day, mean values (± standard deviation) in the same column are not significantly different, $p > 0.05$.
[ABCD]Within each treatment, mean values (± standard deviation) in the same row bearing different superscripts are significantly different, $p < 0.05$.
*$\log_{10}$CFU (colony forming units)/g salmon.

in salmon fillets with enhanced antimicrobial stability.

Previously reported studies, indicate that the antimicrobial activity of seaweeds is dependant on concentration, solvent use and extraction method, as well as the sensitivity of the methods used to determine antimicrobial activity (Cox et al., 2010; Dubber & Harder 2008). Cox et al. (2010) reported methanolic extracts of *P. palmata* to be moderately effective against *Listeria monocytogenes* (62.09%) and weak activity against *Enterococcus faecalis, Pseudomonas aeruginosa* and *Salmonella abony* (39.28, 19.22 and 2.21%, respectively) using the microtitre method. Ethanolic extracts of *P. palmata* increased inhibition of bacteria to 100 and 93.89% against *E. faecalis* and *P. aeruginosa*, respectively. However, no inhibition of 5 fish pathogenic bacteria strains was found using *P. palmata* extracts in the standard agar plate diffusion assay (Bansemir et al., 2006). *In vitro* studies demonstrated antimicrobial activity through the use refined or purified *P. palmata* extracts, however, to date, no scientific literature exists demonstrating antibacterial activity of seaweed or seaweed extracts in food products. In the present study, the lack of antimicrobial activity in the salmon fillets may have been due to purity or level (5-15%) of *P. palmata* incorporated in the salmon feed.

3.3.5 Sensory evaluation of fresh and cooked salmon fillets

In 'visual' sensory analysis, fillets from fresh salmon fed astaxanthin (PC) were very highly significantly positively correlated to pinkness, purchasing appeal and overall acceptability ($p < 0.001$) on days 1 and 7 of storage (Table 3.6). On days 1 and 7 of storage, salmon fed at all levels (0, 5, 10 and 15%) were significantly negatively correlated with pinkness, purchasing appeal and overall acceptability. The observations of the panellists was in agreement with instrumental '*a**' redness values, where PC had the largest '*a**' values compared to all PP treatments. Proximate composition indicated

Table 3.6. Significance of regression coefficients (ANOVA values) of sensory analysis scores for fresh and cooked salmon fillets as derived by jack-knife uncertainty testing.

Parameter	**Storage time, days / Treatment**									
	PC		**0% PP**		**5% PP**		**10% PP**		**15% PP**	
Visual Sensory Analysis	**1**	**7**	**1**	**7**	**1**	**7**	**1**	**7**	**1**	**7**
Pinkness	0.00***	-0.02*	-0.10	0.00***	-0.00***	-0.00***	-0.05*	-0.003**	-0.02*	-0.00***
Whiteness	-0.00***	0.02*	0.10	-0.00***	0.00***	0.00***	0.04*	0.003**	0.01*	0.00***
Drip	-0.28	0.37	-0.55	0.53	0.28	-0.53	0.32	-0.56	0.29	-0.52
Purchasing appeal	0.00***	-0.04*	-0.10	0.00***	-0.00***	-0.00***	-0.06	-0.003**	-0.03*	-0.00***
Overall acceptability	0.00***	-0.03*	-0.10	0.00***	-0.00***	-0.00***	-0.05*	-0.003**	-0.02*	-0.00***
Eating Quality Sensory Analysis										
Colour	0.00***	0.05*	-0.11	-0.00***	-0.00***	-0.00***	-0.07	-0.003**	-0.03*	-0.00***
Texture	0.35	-0.39	-0.35	0.18	-0.36	-0.20	-0.40	-0.21	-0.34	-0.16
Odour	-0.68	0.70	-0.39	0.40	0.68	-0.43	0.69	-0.40	0.69	-0.42
Oxidation flavour	-0.24	0.33	0.39	-0.33	0.24	0.35	0.31	0.36	0.28	0.32
Overall acceptability	0.00***	-0.06	-0.13	0.00***	0.00***	0.00***	-0.08	-0.02*	-0.03*	0.00***

Significance of regression coefficients; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

moisture (~74 to 76 g 100 g^{-1}) was similar across all treatments (Table 3.2) and dietary *P. palmata* did not influence drip loss on days 1 and 7 of storage. Randell et al. (1999) reported drip loss was low (~ 1.5%) in salmon stored in MAP (60% N_2 : 40% CO_2).

In 'eating quality' sensory analysis, PC was very highly significantly positively correlated to colour of cooked salmon fillets while 5% PP was very highly significantly negatively correlated to fillet colour ($p < 0.001$) on both analysis days. After cooking, salmon fillets containing PP appeared whiter than the fresh salmon fillets (5-15% PP). Sensory panellists were able to distinguish between the control and salmon fed PP based on the colour of the cooked salmon fillets. Texture, odour and oxidation flavour in cooked salmon fillets were not significantly influenced by dietary PP. The ability of panellists to detect no oxidation/off flavours in the salmon fillets is in agreement with low levels of lipid oxidation (TBAR) results. On days 1 and 7, salmon fed 5% PP were very highly significantly positively correlated ($p < 0.001$) to overall acceptability (Table 3.6). In a previously reported study, red tilapia (*Oreochromis niloticus*) fed *Spirulina platensis* as the sole source of fish feed did not impact on the taste and smell parameters of the cooked fish fillets (Lu et al., 2003). In the present study, salmon fed low levels of PP (5%) increased overall acceptability, compared to PC, by sensory panellists despite their ability to distinguish between fillets based on colour. Therefore, *P. palmata* incorporated in the salmon feed at low levels (5%) may offer enhancement in overall acceptability with out negatively impacting on texture, odour or oxidation flavours.

3.4 CONCLUSIONS

Salmon fillet surface colour was enhanced with a yellow/orange colour due to deposition of *P. palmata* pigments. Due to consumers growing preference of additives from natural sources, *P. palmata* may prove to be a natural pigment alternative when incorporated into salmon feed and have application in the development of novel functional salmon products. Further research is necessary to investigate if more refined *P. palmata* extracts increase lipid stability. *P. palmata* enhanced overall 'eating quality' acceptability of salmon fillets with comparable product quality (texture, odour and oxidation flavour) and proximate composition to Atlantic salmon (*S. salar*) fed synthetic astaxanthin. Therefore *P. palmata* may be considered as a replacement functional ingredient for farmed Atlantic salmon feed. Further studies will need to examine the use of *P. palmata* with other carotenoid sources to adequately reach final red/pink flesh levels acceptable to consumers.

Porcine studies

CHAPTER 4

Addition of seaweed (*Laminaria digitata*) extracts containing laminarin and fucoidan to porcine diets: Influence on the quality and shelf-life of fresh pork

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ABSTRACT

A seaweed extract containing laminarin (L) and fucoidan (F) (L/F) was manufactured from brown seaweed (*Laminaria digitata*) in spray-dried (L/F-SD) and wet (L/F-WS) forms. The effect of supplementation of pig diets with L/F-SD and L/F-WS (L, 500 mg/kg feed; F, 420 mg/kg feed) for 21 days pre-slaughter, on quality indices of fresh (*longissimus thoracis et lumborum* (LTL)) steaks was examined. Susceptibility of porcine liver, heart, kidney and lung tissue homogenates to iron-induced (1 mM $FeSO_4$) lipid oxidation was also investigated. Dietary supplementation with L/F did not increase plasma total antioxidant status (TAS). In LTL steaks stored in modified atmosphere packs (80% O_2 : 20% CO_2) (MAP) for up to 15 days at 4ºC, muscle pH, surface colour (CIE 'L*' lightness, 'a*' redness and 'b*' yellowness values), lipid oxidation and microbiology (psychrotrophic and mesophilic counts, log CFU/g pork) were unaffected by dietary L/F. A statistically significant reduction in lipid oxidation ($p < 0.05$) was observed in LTL steaks from 75% of pigs ($n = 6$) fed L/F-WS compared to controls. Iron-induced lipid oxidation increased in liver, heart, kidney and lung tissue homogenates over the 24 hr storage period and dietary L/F did not significantly reduced lipid oxidation in organ tissue homogenates.

4.1 INTRODUCTION

Meat and meat products are considered to be a vital component of a healthy diet and important sources of protein, vitamins, minerals and trace elements. In recent years, consumer confidence in meat has been undermined by a number of health concerns related to meat consumption, for example, saturated fat and cholesterol and the associated risk of heart disease, cancer and obesity. Increased consumer demand for healthier meat and meat products, with reduced fat levels, cholesterol, sodium chloride, nitrite, enhanced fatty acid profile and containing health promoting/enhancing ingredients has led to the development of functional meat and meat products (Toldrá & Reig, 2011). The mode of action of functional foods is based on the use of functional ingredients which exert a range of bioactive properties such as antioxidant, anti-inflammatory, anti-cancer and anti-diabetic activities. As concerns regarding the safety and toxicity of synthetic antioxidants grow, the functional properties of many plant extracts have been investigated for their potential use as novel functional ingredients/nutraceuticals.

A number of strategies exist, whereby antioxidants may be incorporated into meat and meat products in order to facilitate the development of functional meats with enhanced health-promoting properties (Khan et al., 2011). Strategies include the supplementation of animal diets with antioxidant compounds or the direct addition of antioxidants to processed meat products. Previous research has focused on dietary supplementation studies, for example, vitamin E (α-tocopheryl acetate), significantly improved the lipid stability of fresh pork (Asghar et al., 1991; Lanari et al., 1995; Monahan et al., 1994). Similarly, antioxidant plant extracts such as tea catechins reduced lipid oxidation in poultry muscle (Tang et al., 2001). Dietary supplementation of oregano oleoresins into porcine diets also reduced lipid oxidation in minced pork (Janz et al., 2007).

Direct addition of plant extracts also reduces lipid oxidation in muscle foods. Carob fruit extracts were found to exhibit antioxidant activity in cooked pork muscle homogenates when added at levels which previously demonstrated bioactivity (reduction in total and LDL cholesterol) in hypercholesterolemic human subjects (Bastida et al., 2009). Lipid oxidation was reduced in pork meat homogenates as a result of the addition cloudberry, beetroot and willow herb (Rey et al., 2005). Addition of tea catechins, rosemary and sage also reduced levels of lipid oxidation in fresh pork patties (McCarthy et al., 2001).

Macroalgae (seaweed) are a potential source of natural antioxidants. Although the nutrient content of seaweed vary with species, geographical location, season and temperature, most contain significant quantities of carbohydrates (polysaccharides, dietary fibre), protein (essential amino acids), lipids (phospholipids), vitamins (ascorbic acid, beta carotene) and minerals (calcium, iron, potassium) (Gupta & Abu-Ghannam, 2011b). Brown seaweeds contain polyphenolic antioxidant compounds such as phlorotannins (phloroglucinol, eckol), catechins (catechin, epigallocatechin, epigallocatechin gallate), tocopherols (α-, γ-, δ-tocopherols), ascorbic acid and carotenoids (α- and β- carotene, fucoxanthin) (Zubia et al., 2009). The vast range of compounds present increases potential use of seaweed and/or extracts in the development of functional foods.

Brown seaweeds are rich in polysaccharides (soluble dietary fibre), the most abundant of which are laminarin, fucoidan and alginic acid. The chemical structure of laminarin (β-glucan) consists mainly of a linear β-(1,3)-linked glucose backbone with some random β-(1,6)-linked glucose side chains depending on the variety of seaweed used for extraction (O'Doherty et al., 2010). Structurally, fucoidan (fucan) is a sulphated polysaccharide containing L-fucose (Costa et al., 2010). Seaweed polysaccharides,

including laminarin and fucoidan are reported to possess antioxidant (Heo et al., 2005), antitumour, antiviral, and antibacterial activities (Costa et al., 2010; O'Doherty et al., 2010; Zubia et al., 2009).

Scientific studies on the potential for incorporating health promoting bioactive compounds derived from seaweed into muscle foods, via supplementation of animal diets, are limited. In addition to deposition of bioactive compounds in muscle tissues of meat producing animals, dietary bioactive compounds also demonstrate potential to improve animal health and welfare. Supplementation of pig diets with laminarin and fucoidan (isolated from *Laminaria digitata*) has previously been shown to improve growth performance and gut health in pigs (O'Doherty et al., 2010).

The objective of this study was to assess the effect of dietary supplementation of porcine diets with a seaweed extract containing laminarin and fucoidan (L/F), isolated from *Laminaria digitata*, on the plasma antioxidant status, muscle pH, colour, lipid oxidation and microbiology of fresh *longissimus thoracis et lumborum* (LTL) steaks stored in modified atmosphere packs (MAP) at 4ºC. The influence of dietary L/F on iron-induced lipid oxidation in porcine liver, heart, kidney and lung tissue homogenates was also examined.

4.2 MATERIALS AND METHODS

4.2.1 Reagents

All chemicals used were 'AnalaR' grade obtained from Sigma-Aldrich Ireland Ltd., Arklow, Co. Wicklow, Ireland, Oxoid Ltd., Basingstoke, Hampshire, England and Merck KGaA, Darmstadt, Germany. The total antioxidant status (TAS) Randox-Trolox kit was obtained from Randox Laboratories Ltd., Co. Antrim, UK. A seaweed extract (L/F), containing laminarin (L) and fucoidan (F) was manufactured by Bioatlantis, Tralee, Co. Kerry, Ireland. The extract isolated from brown seaweed (*Laminaria digitata*), harvested in Ireland, was prepared using an acid extraction technique, details of which are confidential.

4.2.2 Animals and diets

Twenty four pigs (large white x landrace cross consisting of 12 males and 12 females) (average live weight ~ 14.51 kg, age: 7 weeks) were randomly assigned to one of three treatments (n = 8) and fed *ad libitum* for 21 days pre-slaughter following a completely randomised experimental design. The control group (Control) were fed a basal diet (Table 4.1). The second group were fed the basal diet plus a spray-dried seaweed extract containing laminarin and fucoidan (L/F-SD) at an inclusion rate of 5.37 kg/tonne of feed. The third group were fed the basal diet plus a wet formulation of the seaweed extract containing laminarin and fucoidan (L/F-WS) at an inclusion rate of 26.3 kg/tonne of feed. Inclusion rates are based on the laminarin and fucoidan content of the spray-dried (L/F-SD) and wet (L/F-WS) seaweed extracts. Therefore both treatment groups received diets containing L, 500 mg/kg feed and F, 420 mg/kg feed.

The composition of the experimental diet and the seaweed extracts containing L/F are outlined in Tables 4.1 and 4.2, respectively. Dry matter, crude protein, neutral

Table 4.1. Composition and chemical analysis of the experimental diet (g/kg, unless otherwise indicated).

Composition	**Basal Diet**[a]
Wheat	660.40
Soya-bean meal	260.00
Soya oil	24.80
Minerals and Vitamins[b]	23.00
Lysine HCL	3.40
L-Threonine	1.30
DL-Methionine	0.80
Analysed Composition	
Dry Matter	887.70
Crude protein (N*6.25)	186.60
Neutral Detergent Fibre	115.30
Ash	46.50
Gross Energy (MJ/kg)	17.30
Calcium[†]	6.90
Phosphorus[†]	4.35
Lysine[†]	10.00
Methionine and cysteine[†]	6.00
Threonine[†]	6.50
Tryptophan[†]	1.80

[a]Control group - basal diet; L/F-SD - basal diet supplemented with 5.37 kg/tonne of spray-dried seaweed extract containing L/F and 20.9 kg water; L/F-WS - basal diet supplemented with 26.3 kg/tonne of wet seaweed extract containing L/F.
[b]Vitamin and mineral inclusion (per kg diet): 3 mg retinol, 0.05mg cholecalciferol, 40 mg α-tocopherol, 25 mg copper as copper II sulphate, 100 mg iron as iron II sulphate, 100 mg zinc as zinc oxide, 0.3 mg selenium as sodium selenite, 25 mg manganese as manganese oxide and 0.2 mg iodine as calcium iodate on a calcium sulphate/ calcium carbonate carrier.
[†]Calculated from tabulated nutritional composition (Sauvant, Perez, & Tran, 2004).

detergent fibre, ash and gross energy analysis of the experimental diet was carried out as described by Leonard et al. (2011). The laminarin content (%) of the spray-dried (L/F-SD) and wet (L/F-WS) seaweed extracts was measured using a commercial assay kit (Megazyme International Ireland, Bray, Co. Wicklow, Ireland). Fucoidan levels (%) in L/F-SD and L/F-WS were determined as described by Usov et al. (2001).

The animals were housed individually (1.68 m × 1.22 m slatted pens) at Lyons Research Farm, University College Dublin, Newcastle, Co. Dublin, Ireland and fed *ad libitum* from hopper style feeders. Water was supplied *ad libitum* from individual nipple

drinkers. The average daily feed intake was 1.8 kg/day for the control and L/F-WS groups, and 1.9 kg/day for the L/F-SD group (SEM 0.09). The ambient environmental temperature within the houses was thermostatically controlled and maintained at 22°C. The pigs were slaughtered at the end of the feeding period via euthanol injection (pentobarbitone sodium patent blue) (injection rate: 1 ml/1.4 kg live weight) by veterinary personnel. A blood sample for plasma analysis was taken immediately following injection. The mean slaughter weight was 29.7 kg. The liver and kidneys were removed and frozen in a blast freezer. Carcasses and organs were transported at 4ºC to the School of Food and Nutritional Sciences at University College Cork, Ireland where they were hung at 2ºC for 24 hrs. Liver and kidney tissues were placed in vacuum pack bags (composed of polyamide and a polyethylene sealing layer), vacuum packed and stored at -18ºC for four months prior to analysis. Blood samples were centrifuged to separate the plasma fractions and stored at -20ºC prior to analysis.

Table 4.2. Composition of the seaweed extracts containing laminarin and fucoidan.

	L/F-SD	**L/F-WS**
% Total Solids	94.03	18.5
% Ash[a]	68.6	13.4
% Protein	0.64	0.12
% Laminarin	9.3	1.9
% Fucoidan	7.8	1.6
% Mannitol	8.3	1.62
Inclusion rate (per tonne feed)	5.37 Kg	26.3 Kg

[a]The ash content of the seaweed extract was 15 g/kg DM Ca, 10 g/kg DM Na, 10 g/kg DM K, 10 g/kg DM S, 250 mg/kg DM iodine, 250 mg/kg DM Fe, 20 mg/kg DM Cu and 50 mg/kg DM Zn.

4.2.3 Plasma total antioxidant status

Blood samples (10 ml) were collected by jugular veni-puncture using vacutainers containing lithium/heparin as anti-coagulant (Becton Dickinson, Rutherford, NJ, USA) from all animals immediately after slaughter. The blood was centrifuged (Beckman J2-

21, Beckman Instruments Inc., CA, USA) at 4720g for 20 min at 4ºC. The plasma layer was removed from the red blood cell layer and stored at -18 ºC for subsequent analysis.

The total antioxidant status (TAS) of porcine blood plasma was measured according to the manufacturers' instructions. Plasma (20 µl) was added to 1 ml chromogen (metmyoglobin/ABTS®) and mixed thoroughly. Tubes were placed in a water bath at 37°C for 10 min. An initial absorbance reading of the coloured complex was recorded after 10 min using a spectrophotometer (Cary 300 Bio, UV-Vis spectrophotometer, Varian Instruments, CA, USA) at 600 nm against a blank containing all reagents and double de-ionised water. Tubes were returned to the water bath and 200 µl of substrate (hydrogen peroxide in stabilised form) was added. A second absorbance measurement of the colour complex was recorded 3 min following substrate addition. The antioxidant activity was calculated using the following equation:

$$\text{mmol/l} = \text{Factor} \times (\Delta A \text{ Blank} - \Delta A \text{ Sample})$$

$$\text{Factor} = (2.04 \text{ mmol/l}) / (\Delta A \text{ blank} - \Delta A \text{ standard})$$

Total antioxidant status (TAS) was expressed as mmol of trolox equivalent antioxidant capacity (TEAC)/L plasma.

4.2.4 Pork processing and packaging

Following storage at 2ºC for 24 hrs, l*ongissimus thoracis et lumborum* was excised from each carcass, vacuum packed and stored at 2ºC for 24 hrs. The heart and lungs were removed from each carcass, vacuum packed and stored at -18ºC with the previously blast-frozen liver and kidney tissues.

Longissimus thoracis et lumborum (LTL) were cut into steaks (~ 1inch thickness, ~ 45 g portion), placed in low oxygen permeable (<1cm^3/m^2/24 h at STP) polystyrene/ethylvinylalcohol (EVOH)/polyethylene (PE) trays. Trays were covered

using a low oxygen permeable (3 cm^3/m^2/24 h at STP) laminated barrier film with a polyolefin heat sealable layer and flushed with 80% O_2 : 20% CO_2 (modified atmosphere packs, MAP) using a vacuum-sealing unit (VS 100, Gustav Müller & Co. KG, Homburg, Germany) equipped with a gas mixer (Witt-Gasetechnik GmbH & Co. KG, Witten, Germany) and heat-sealed. LTL steaks in MAP were stored for up to 15 days under fluorescent lighting (660 lux) at 4ºC. The gas atmosphere in the MAP was checked using a CheckMate 9900 (PBI-DanSensor, Denmark). Immediately after gas flushing, MAP contained 70.18 ± 0.33% O_2 and 23.14 ± 0.31% CO_2. The average gas composition in MAP at the end of the 14 day storage period was 68.43 ± 1.02% O_2 and 23.88% ± 0.59% CO_2.

4.2.5 Measurement of pH

Pork samples (10 g) were homogenised for 1 min at 24,000 rpm in 90 ml distilled water using an Ultra Turrax T25 homogeniser (Janke and Kunkel, IKA-Labortechnik, GmbH and Co., Staufen, Germany). The pH of the pork homogenates was measured at 20°C using a pH meter (Seven Easy portable, Mettler-Toledo GmbH, Schweizenbach, Switzerland). The pH of LTL steaks was recorded on days 1, 4, 7, 11 and 14 of storage.

4.2.6 Colour measurement

The surface colour was measured using a Minolta CR-300 Chroma Meter (Minolta Camera, Co., Osaka, Japan). The Chroma Meter consisted of a measuring head (CR-300), with an 8 mm diameter measuring area, a 2° standard observer, and a data processor (DP-301). The chroma meter was calibrated on the CIE LAB colour space system using a white tile (D_c:L = 97.79, a = -0.11, b = 2.69). The 'L*' value represents

lightness and '*a**' and '*b**' values represent redness and yellowness, respectively. Colour measurements of LTL steaks were recorded on days 1, 4, 7, 11 and 14 of storage.

4.2.7 Measurement of lipid oxidation

Lipid oxidation was measured using the 2-thiobarbituric acid assay of Siu and Draper (1978). Chopped pork samples (5 g) were homogenised for 2 min in 25 ml distilled water using an Ultra Turrax tissue homogeniser. Trichloroacetic acid (10%) was added (25 ml) and the mixture was shaken vigorously and filtered through Whatman No. 1 filter paper. In screw capped test tubes, 4 ml of clear filtrate was added to 1 ml of 0.06 M 2-thiobarbituric acid (TBA). The tubes were placed in a water bath and held at 80°C for 90 min. The absorbance of the filtrate was measured spectrophotometrically (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 of 0.06 M TBA reagent). The malondialdehyde content of the sample 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 pork. Lipid oxidation in LTL steaks was measured on days 1, 4, 7, 11 and 14 of storage.

4.2.8 Microbiological analysis

Pork (10 g) was transferred into stomacher bags, diluted with 90ml of maximum recovery diluent and stomached for 3 min (Steward Stomacher 400 Lab Blender, London, UK) resulting in a 10^{-1} dilution used for analysis. Serial dilutions were prepared and 0.1 ml aliquots from each dilution were plated onto standard plate count agar (PCA) (Oxoid Ltd.). The plates were incubated at 30°C for 48 hrs and at 4°C for 10 days to determine mesophilic and psychrotrophic counts, respectively. Microbiological analysis of LTL

steaks was carried out on days 1, 5, 8, 12 and 15 of storage. Results were expressed as log_{10}CFU (colony forming units)/g pork.

4.2.9 Preparation of liver, heart, kidney and lung tissue homogenates

Liver, heart, kidney and lung tissue homogenates (25%) were prepared by homogenising 20 g tissue in 60 ml 0.12M KCl 5 mM histidine (pH 5.5) using an Ultra Turrax T25 homogeniser at 24,000 rpm for 3 min. A stock solution (30 ml) containing 30mM $FeSO_4$ was prepared in distilled water. Stock solution (1 ml) was added to 29 ml of tissue homogenate resulting in a final $FeSO_4$ concentration of 1 mM $FeSO_4$. Lipid oxidation in 30 ml tissue homogenate samples, held in 150 ml beakers at 4°C, was initiated by the addition of 1 mM $FeSO_4$. Homogenates without $FeSO_4$ were run simultaneously as controls. Lipid oxidation was measured immediately (time 0) and after 24 hrs storage at 4°C.

4.2.9.1 Measurement of lipid oxidation in organ tissue homogenates

A modification of the 2-thiobarbituric acid (TBA) assay of Siu and Draper (1978) was used to measure lipid oxidation in liver, heart, kidney and lung tissue homogenates. Homogenate samples (4 ml) were added to 4 ml 10% trichloroacetic acid (TCA). The samples were mixed using a vortex mixer and the precipitate formed was removed by filtering through Whatman No. 1 filter paper. In a screw cap test tube, 4 ml of clear supernatant was added to 1 ml 0.06 M 2-thobarbituric acid (TBA). The tubes were incubated at 80°C for 90 min and the absorbance of the resulting coloured complex was measured using a spectrophotometer at 532 nm against a blank containing all reagents and distilled water instead of the filtrate. Tubes containing supernatant from filtered liver homogenates and 10% TCA, incubated with TBA reagent, were centrifuged at 1000g,

prior to absorbance measurements to pellet a precipitate formed during the heating step. The malondialdehyde content was calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. Results were expressed as TBA reactive substances (TBARS) in mg malondialdehyde (MDA)/kg organ tissue.

4.2.10 Statistical analysis

All analyses were performed in duplicate. Mean sample values (n = 8) for each of the three treatment groups (Control, L/F-SD and L/F-WS) were subjected to statistical analysis. A full repeated measures ANOVA was conducted to investigate the effects of dietary L/F form (spray-dried (L/F-SD) and wet (L/F-WS) forms), time and their interactions. Dietary L/F form represented the 'between-subjects' factor and the effect of time was measured using the 'within-subjects' factor. Tukey's test was used to adjust for multiple comparisons between treatment means. The analysis was carried out using the SPSS 18.0 for Windows (SPSS, Chicago, IL, USA) software package.

4.3 RESULTS AND DISCUSSION

4.3.1 Plasma total antioxidant status and muscle pH

In previously reported studies, antioxidant compounds have been detected in the plasma of animals and humans following ingestion. Detection of antioxidant compounds in blood plasma is a useful indicator of the fate of supplemental extracts and suggests that compounds or their metabolites are readily bio-available for potential uptake into muscle tissues. The consumption of foods rich in phenolic antioxidants (strawberries, spinach or red wine) has been found to significantly increase the antioxidant status of plasma in humans (ORAC, TEAC and FRAP assays) (Cao et al., 1998). In rat plasma elevated tea catechin concentrations have been reported following oral administration of a single dose (500 mg/kg body weight) (Nakagawa & Miyazawa, 1997). Antioxidants such as vitamin E (α-tocopherol) have been detected in porcine plasma with levels increasing 2.5-fold after approximately 7 days of supplementation (200 mg/kg feed) (Morrissey et al., 1996).

In the present study, the total antioxidant status (TAS) of porcine plasma was determined in order to assess the effectiveness of dietary supplementation with L/F. The average TAS was 0.55, 0.67 and 0.60 mmol TEAC/L plasma for the control group, L/F-SD and L/F-WS, respectively. Porcine plasma TAS was not significantly affected by L/F supplementation compared to the control group. Gladine et al. (2007a) reported no increase in plasma TAS in rats fed plant extracts (rosemary, grape, citrus and marigold, 0.5 g/kg diet for three weeks), however a significant reduction in plasma lipid oxidation (malondialdehyde (MDA)) was observed. While laminarin and fucoidan have strong antioxidant capacities *in vitro* (Heo et al., 2005; Wang et al., 2010a), to date limited research exists to support the contention that consumption of polysaccharides augments plasma antioxidant levels *in vivo*. Gladine et al. (2007a) reported no correlation between *in vitro* and *in vivo* antioxidant capacity of plant extracts rich in polyphenols and further

suggested that bioavailability efficiency and TAS are more a general indicator of plasma oxidative stability rather than specifically reflecting lipid susceptibility to oxidation.

The reason for the lack of an effect of dietary L/F on the TAS of porcine plasma is unclear. In a previously reported study which examined the absorption of dextran sulphate (branched glucan) (MW about 8000), administered orally in a short-term (single dose, 4 g/day for 5 days) and long term (1 g, 4 times/day for 29 to 335 days) study, dextran sulphate was detected in high concentrations in endothelial cells and at low levels in plasma as early as 2.4 min after administration. It was concluded that dextran sulphate was extensively absorbed and rapidly incorporated into the endothelium therefore plasma levels were negligible (Hiebert et al., 1999). By contrast, a single dose (0.1 ml/10 g of body weight) of α-D-glucans from fungi (*Tricholoma matsutake*) administered to mice, resulted in detection of α-D-glucans in mice plasma 16 hrs following oral administration. Maximum α-D-glucans levels were detected by 24 hrs, after which, levels declined up to 48 hrs (Hoshi et al., 2008). Previous literature reported that distribution of glucans in the body is affected by various factors, such as molecular weight, particle size, fine structure, charge, association, and susceptibility to enzymatic hydrolysis (Hiebert et al., 1999). In the present study, pigs were fed up to 3 hrs prior to slaughter, therefore rapid absorption of L/F metabolites by the GI tract may account for the lack of an effect of dietary L/F on the total antioxidant status (TAS) of porcine plasma.

The pH of fresh LTL muscle ranged from 5.90 to 5.19 over the 15 day storage period and was unaffected by L/F supplementation of porcine diets. pH values reported are comparable to previously reported values (5.8-5.4) for post-mortem muscle (Faustman & Cassens, 1990).

4.3.2 Colour stability of fresh LTL muscle

The surface lightness '*L**' significantly ($p < 0.05$) increased in fresh LTL muscle over the 14 day storage period. Surface '*a**' redness values significantly ($p < 0.05$) decreased as a function of storage time (Table 4.3). Each colour parameter was not affected by L/F supplementation. Many studies have provided evidence that pigment (oxymyoglobin) oxidation and lipid oxidation are closely interrelated where an increase in one results in a similar increase in the other. This is potentially due to decreased dissolved oxygen levels or radicals produced as a result of the lipid oxidation process (O'Grady et al., 2001). Levels of lipid oxidation were relatively low over the 14 day storage period. Therefore protection against pigment oxidation due to L/F supplementation, mediated through a reduction in lipid oxidation, was not evident in the present study.

While some previously reported studies indicate that colour stability of pork muscles may be improved when antioxidants are incorporated into animal diets, others demonstrate no effect of dietary antioxidants on fresh pork colour stability. For example, in pigs fed vitamin E (α-tocopheryl acetate), some studies report positive benefits of feeding vitamin E on pork colour stability, while others report no beneficial effects on fresh pork colour (Asghar et al., 1991; Houben et al., 1998; Lanari et al., 1995; Monahan et al., 1994). Similar to results reported in the present study, oat-based diets containing β-glucans (1.6, 2.1, 3.3 or 4.1%) did not influence the colour stability of porcine *longissimus* muscle (Fortin et al., 2003) while other plant extracts (Melissa, Origanum and salvia) (10 ml/pig for 10 days) improved muscle colour stability when incorporated into porcine diets (Lahucky et al., 2010). In the studies reported, variations in the efficacy of dietary antioxidants on the colour stability of pork meat may also be attributed to differences in factors such as storage temperature and packaging treatment.

Table 4.3. Effect of dietary laminarin/fucoidan (L/F) on the surface lightness ('*L**' value), redness ('*a**' value) and yellowness ('*b**' value) values of fresh *longissimus thoracis et lumborum* (LTL) stored in modified atmosphere packs (80% O_2 : 20% CO_2) for up to 14 days at 4°C.

Treatment		**Storage time at 4°C, days**				
	Parameter	**1**	**4**	**7**	**11**	**14**
Control	Lightness	58.20 ± 0.70^{aA}	60.74 ± 1.13^{aAB}	61.47 ± 1.35^{aAB}	62.15 ± 1.42^{aAB}	63.63 ± 1.68^{aB}
L/F-SD	*L**	59.04 ± 1.98^{aA}	60.63 ± 1.23^{aAB}	62.12 ± 1.42^{aAB}	62.06 ± 1.55^{aAB}	63.39 ± 1.55^{aB}
L/F-WS		58.09 ± 1.53^{aA}	59.69 ± 1.42^{aAB}	60.63 ± 0.92^{aABC}	61.73 ± 1.09^{aBC}	63.17 ± 1.54^{aC}
Control	Redness	9.00 ± 0.90^{aA}	8.95 ± 0.88^{aAB}	8.65 ± 1.11^{aAB}	7.42 ± 0.78^{aBC}	5.96 ± 1.15^{aC}
L/F-SD	*a**	10.35 ± 1.06^{aA}	8.88 ± 0.81^{aAB}	8.26 ± 1.18^{aAB}	7.16 ± 0.95^{aBC}	5.72 ± 0.81^{aC}
L/F-WS		10.06 ± 1.09^{aA}	9.34 ± 0.82^{aAB}	8.07 ± 0.77^{aBC}	7.90 ± 0.63^{aBC}	6.44 ± 0.93^{aC}
Control	Yellowness	7.35 ± 0.80^{aAB}	8.02 ± 0.61^{aA}	8.02 ± 0.72^{aA}	8.03 ± 0.81^{aA}	9.36 ± 0.80^{aB}
L/F-SD	*b**	8.24 ± 0.87^{aA}	8.35 ± 0.63^{aA}	8.52 ± 0.69^{aA}	8.46 ± 0.73^{aA}	8.77 ± 0.85^{aA}
L/F-WS		8.21 ± 0.64^{aA}	8.35 ± 0.59^{aA}	7.59 ± 0.56^{aA}	8.21 ± 0.61^{aA}	8.40 ± 0.48^{aA}

[a]Within each parameter and storage day, mean values (± standard deviation) in the same column are not significantly different, $p > 0.05$.
[ABC]Within each treatment, mean values (± standard deviation) in the same row bearing different superscripts are significantly different, $p < 0.05$.

Interactions between polysaccharides and proteins have previously been reported in the scientific literature however, knowledge of the role of these interactions in relation to functionality in complex multiphasic systems is limited (Doublier et al., 2000). The lack of an effect of dietary L/F on the colour stability of pork (*a** redness values) indicates no interaction between polysaccharides (L/F) and proteins (oxymyoglobin) in the present study. In addition, following ingestion and absorption, polysaccharides are potentially bio-transformed into different forms.

4.3.3 Lipid oxidation in fresh LTL muscle

Lipid oxidation significantly ($p < 0.05$) increased in all groups over the 14 day storage period. Overall, the levels of lipid oxidation were low with mean values ranging from 0.12-1.70 mg MDA/kg pork. Lowest levels of oxidation were observed in LTL steaks from pigs fed the wet supplement (L/F-WS), although results were not statistically significant ($p > 0.05$) (Table 4.4). Although not significant, trends for lipid oxidation followed the order: C > LF-SD > L/F-WS. The spray-drying process during the manufacture of L/F-SD may have negatively affected and lowered the antioxidant capacity of the L/F supplement. While trends show a positive effect on limiting lipid oxidation in both the L/F supplemented diets, a statistically significant effect on levels of lipid oxidation was observed in the L/F-WS group when 75% of pigs ($n = 6$) were compared to the control group ($p < 0.05$) (data not shown). Therefore, it was concluded that antioxidant components of laminarin and fucoidan were deposited in porcine muscle (LTL) following the inclusion of L/F in animal diets.

Many *in vitro* antioxidant assays (DPPH, FRAP, TEAC, NO, ABTS radical scavenging) have demonstrated antioxidant potential of numerous seaweed polysaccharides (Ngo et al., 2011) including sulphated polysaccharides and β-glucans

Table 4.4. Effect of dietary laminarin/fucoidan (L/F) on lipid oxidation (TBARS*) in fresh *longissimus thoracis et lumborum* (LTL) stored in modified atmosphere packs (80% O_2 : 20% CO_2) for up to 14 days at 4°C.

Treatment	Storage time at 4°C, days				
	1	4	7	11	14
Control	0.19 ± 0.04^{aA}	0.24 ± 0.15^{aA}	0.30 ± 0.12^{aA}	0.78 ± 0.51^{aA}	1.70 ± 1.02^{aB}
L/F-SD	0.16 ± 0.03^{aA}	0.19 ± 0.05^{aA}	0.28 ± 0.12^{aAB}	0.94 ± 0.92^{aBC}	1.21 ± 0.70^{aC}
L/F-WS	0.13 ± 0.02^{aA}	0.12 ± 0.03^{aA}	0.19 ± 0.04^{aAB}	0.48 ± 0.16^{aB}	0.91 ± 0.45^{aC}

[a]Within each storage day, mean values (± standard deviation) in the same column are not significantly different, $p > 0.05$.
[ABC]Within each treatment, mean values (± standard deviation) in the same row bearing different superscripts are significantly different, $p < 0.05$.
*TBARS, mg malondialdehyde (MDA)/kg pork.

from a variety of seaweeds (*Turbinaria conoides*, *Laminaria japonica*, *Sargassum fulvellum*, *Dictyota mertensii*) (Costa et al., 2010; Ngo et al., 2011; Paiva et al., 2011; Wang et al., 2010a). Limited research exists linking the antioxidant activity of seaweed extracts *in vitro* with *in vivo* studies in human or animal subjects (in particular pigs).

Seaweeds have long been used as soil fertilisers and in animal feeds for many years but historically much of the information has been subjective (Fike et al., 2001). A previous study which examined the effects of a seaweed (*Ascophyllum nodosum*)-based proprietary product on tall fescue (bunch grasses), reported that grass fertilised with the seaweed product improved antioxidant activity in grasses and subsequently increased the antioxidant activity of ruminant animals grazed on the treated grasses by increasing serum vitamin A and whole-blood selenium levels (Fike et al., 2001). In the present study, direct supplementation of L/F had an impact on lowering levels of lipid oxidation in porcine muscle, however results were not statistically significant ($p > 0.05$).

4.3.4 Lipid oxidation in porcine liver, heart, kidney and lung tissue homogenates

The uptake and distribution of dietary antioxidant compounds such as vitamin E has been measured in various porcine tissues (Morrissey et al., 1996). Due to the chemical nature and complexity of the L/F seaweed extracts utilised in the present study,

the LTL muscle and tissues examined were not assayed directly for laminarin and fucoidan present in the seaweed extract. In order to determine whether antioxidant compounds present in the L/F extract were distributed throughout porcine tissues, other than the LTL muscle, liver, heart, kidney and lung tissue homogenates were subjected to iron ($FeSO_4$)-induced lipid oxidation. Following $FeSO_4$ addition, lipid oxidation increased in all tissue homogenates over the 24 hr storage period. Trends indicate lower levels of lipid oxidation in liver tissue homogenates, as a result of dietary L/F, however due to variation in results were not statistically significant (Table 4.5). In heart, kidney and lung tissue homogenates, significant decreases in levels of lipid oxidation were not detected.

Table 4.5. Effect of dietary laminarin/fucoidan (L/F) on iron-induced lipid oxidation (TBARS*) in organ (liver, heart, kidney and lung) tissue homogenates after 24 hours storage at 4ºC.

Treatment	**Storage for 24 hours at 4ºC**			
	liver	**heart**	**kidney**	**lung**
Control	3.20 ± 1.43^a	3.26 ± 0.26^a	3.06 ± 1.01^a	2.99 ± 0.93^a
L/F-SD	1.99 ± 1.54^a	3.34 ± 0.27^a	2.26 ± 1.09^a	2.27 ± 1.82^a
L/F-WS	1.46 ± 1.36^a	3.28 ± 0.27^a	2.59 ± 0.52^a	2.96 ± 1.30^a

[a]Within each organ type, mean values (± standard deviation) in the same column are not significantly different, $p > 0.05$.
*TBARS, mg malondialdehyde (MDA)/kg organ tissue.

In a previously reported dietary study, where rats were fed plant extracts (rosemary, grape, citrus and marigold, 0.5 g/kg diet for three wks) with bio-efficiency in the liver, it was concluded that supplementation for 3 wks was not sufficient to significantly modify the intensity of lipid oxidation in extra-hepatic tissues (*longissimus thoracis* and heart muscles) (Gladine et al., 2007b). In a related study, dietary laminarin extracted from brown algae, fed for 25 days (5% during 4 days followed by 10% during 21 days) was found to modulate intra-hepatic immune cells in rats thus protecting the liver from damage (Neyrinck et al., 2007). Such findings indicate deposition of laminarin

in liver tissues following oral ingestion. Similarly, Airanthi et al. (2011) reported that mice, fed brown seaweed extracts (*Undaria pinnatifida*, *Sargassum horneri*, and *Cystoseira hakodatensis*) (0.5% and 2%) for four weeks, had significantly lower levels of lipid hydroperoxides in liver tissue homogenates compared to controls. Supplementation of rat diets with seaweed powder (*Eucheuma cottonii*) (5% and 10% for 35 days) increased the activity of superoxide dismutase (antioxidant enzyme) in liver tissues of hypocholesterolemic rats (Wresdiyati et al., 2009).

4.3.5 Microbiology of fresh LTL muscle

The mesophilic plate counts ranged from 4.32-4.58 log cfu/g on day 1 and increased to a maximum of 7.41 log cfu/g on day 15 of storage (Table 4.6). Psychotropic plate counts ranged from 3.24-3.42 log cfu/g on day 1 and increased to levels ranging from 7.94-8.20 log cfu/g on day 15 (Table 4.6). Mesophilic counts obtained are similar to previously reported values for fresh pork (Houben et al., 1998). Previously reported benefits of L/F include antimicrobial activity in test systems such as growth inhibition assays and the agar plate diffusion test, however results from the present study indicate that L/F dietary supplementation did not exert antimicrobial activity in fresh pork muscle.

Several *in vitro* antimicrobial assays demonstrate antimicrobial activities of various types of seaweed including extracts from *Ceramium rubrum*, *Mastocarpus stellatus* and *Laminaria digitata*. An extract from *Laminara digitata* (31 mg dry weight/ml) demonstrated strong antibacterial activity and inhibited almost all test bacteria (12 marine and 7 prominent fish pathogenic bacteria). In addition, *Laminaria digitata* resulted in both bacteriostatic and bacteriolytic (the destruction of bacteria by lysis) modes of action (Dubber & Harder, 2008). While antimicrobial activity of seaweed extracts has been reported using *in vitro* assays, to date, the scientific literature contains

Table 4.6. Effect of dietary laminarin/fucoidan (L/F) on the microbial status* (mesophilic and psychrotrophic) of fresh *longissimus thoracis et lumborum* (LTL) stored in modified atmosphere packs (80% O_2 : 20% CO_2) at for up to 15 days at 4°C.

Treatment		**Storage time at 4°C, days**				
	Temp	**1**	**5**	**8**	**12**	**15**
Control	30°C	4.32 ± 0.20^{aA}	4.72 ± 0.36^{aA}	6.52 ± 0.48^{aB}	6.99 ± 0.38^{aBC}	7.37 ± 0.05^{aC}
L/F-SD	(mesophilic	4.49 ± 0.18^{aA}	4.95 ± 0.14^{aA}	6.50 ± 0.57^{aB}	6.95 ± 0.56^{aB}	7.27 ± 0.50^{aB}
L/F-WS	count)	4.58 ± 0.10^{aA}	4.93 ± 0.36^{aA}	6.32 ± 0.38^{aB}	7.47 ± 0.14^{aC}	7.41 ± 0.35^{aC}
Control	4°C	3.24 ± 0.48^{aA}	5.68 ± 0.81^{aB}	7.23 ± 0.19^{aC}	7.88 ± 0.32^{aCD}	8.15 ± 0.25^{aD}
L/F-SD	(psychrotrophic	3.34 ± 0.11^{aA}	5.51 ± 0.59^{aB}	7.05 ± 0.21^{aC}	7.99 ± 0.36^{aD}	8.20 ± 0.23^{aD}
L/F-WS	count)	3.42 ± 0.32^{aA}	5.69 ± 0.37^{aB}	7.29 ± 0.14^{aC}	8.06 ± 0.19^{aD}	7.94 ± 0.26^{aD}

[a]Within each storage temperature and day, mean values (± standard deviation) in the same column are not significantly different, $p > 0.05$.
[ABCD]Within each treatment, mean values (± standard deviation) in the same row bearing different superscripts are significantly different, $p < 0.05$.
*$\log_{10}$CFU (colony forming units)/g pork.

no studies demonstrating antibacterial activity of seaweed extracts in food products (Gupta & Abu-Ghannam, 2011b).

4.4 CONCLUSIONS

Addition of L/F to porcine diets did not affect the surface colour of pork meat. No significant antioxidant or antimicrobial effect of dietary L/F on pork meat was observed under the experimental conditions employed in the present study. Due to concerns regarding toxicity of synthetic antioxidants, L/F may prove to be a natural antioxidant alternative when incorporated into animal feed. Further research is necessary to examine the effects of dietary laminarin and fucoidan levels, form and duration of feeding on the quality and shelf-life of fresh pork.

CHAPTER 5

Influence of level and duration of feeding polysaccharide (laminarin and fucoidan) extracts from brown seaweed (Laminaria digitata) on quality indices of fresh pork

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ABSTRACT

The effect of level (450 or 900 mg laminarin (L) and fucoidan (F) /kg feed) and duration (3 or 6 weeks) of feeding a seaweed (*Laminaria digitata*) extract containing L/F on the quality of pork (*longissimus thoracis et lumborum* (LTL)) stored in modified atmosphere packs and on organ lipid stability was examined. Mechanisms of L/F antioxidant activity in LTL were evaluated. Plasma total antioxidant status, LTL pH, colour, microbiology and 'eating quality' sensory analysis were unaffected by dietary L/F. 'Visual' sensory descriptors (purchasing appeal and overall visual acceptability) were enhanced ($p < 0.05$) in $L/F_{450 - 3}$ LTL. Lipid oxidation was lower ($p < 0.05$) in $L/F_{450 - 3}$ and $L/F_{900 - 3}$ LTL (on day 11 and 14 of storage). In cooked minced pork, lipid oxidation was not significantly reduced by dietary L/F. Saturated fatty acids were lower ($p < 0.05$) in $L/F_{900 - 6}$ LTL. Results indicated L/F in pig diets for 3 weeks enhanced lipid stability of pork meat.

5.1 INTRODUCTION

The use of antioxidants in the meat industry is an effective way to minimize or prevent lipid oxidation, retard the formation of toxic oxidation products, maintain nutritional quality and prolong shelf-life (Gupta & Abu-Ghannam, 2011b). The addition of antioxidants to meat and meat products can be achieved by supplementing animal diets with specific compounds for a defined duration pre-slaughter. The level and duration of dietary antioxidant compounds necessary to enhance meat quality parameters is dependent on factors such as chemical structure and purity of the antioxidant, animal species, breed and other physiological factors. Previous strategies have investigated various levels and durations of feeding antioxidant compounds such as α-tocopheryl acetate (vitamin E), β-carotene, ascorbic acid, tea catechins, chitosan and plant extracts to pigs for the determination of an optimum level and duration combination required to enhance pork meat quality (Decker et al., 2000).

Supplementation of α-tocopheryl acetate in porcine diets has proven effective in enhancing the oxidative stability of *longissimus dorsi* muscle which increased as a function of dietary level (20 and 200 mg/kg feed) and duration (0, 35, 126 days) of supplementation, in post mortem muscle (Morrissey et al., 1996). Similarly, dietary green tea catechins (200 mg/kg) lowered levels of lipid oxidation in *longissimus thoracis et lumborum* (LTL) pork steaks (Mason et al., 2005). Lahucky et al. (2010) reported that dietary supplementation of oregano extract (30 and 60 ml/day) in pig diets increased lipid stability in *longissimus thoracis* muscle as a function of level, however the same dose response was not observed with dietary supplementation of Melissa (20 and 100 ml/day).

The potential of dietary antioxidants to influence other quality parameters such as the colour stability of pork meat varies considerably (Jensen et al., 1998). Buckley et al. (1995) reported an increase in '*a**' redness values of LTL muscle from pigs fed a high

level of α-tocopheryl acetate (200 mg/kg feed) compared to pigs fed a basal level of 10 mg α-tocopheryl acetate /kg of feed. Conversely, Jensen et al. (1997) reported no influence on the colour stability of LTL pork steaks when pigs were fed increasing levels of α-tocopheryl acetate (100, 200 and 700 mg/kg feed). Similarly, Mason et al. (2005) reported green tea catechins (200 mg/kg) did not enhance the colour stability of LTL pork steaks. However, supplementation with ascorbic acid (0, 100, 250 or 500 mg/L) in pig diets enhanced the '*a**' redness values of LTL muscle at the highest level (500 mg/L) (Pion et al., 2004).

Due to their complex structure, seaweed polysaccharides have diverse biological properties (Thomes et al., 2010). Bioactive compounds identified in seaweed extracts have demonstrated a capacity to neutralize superoxide and hydroxyl radicals (Bocanegra et al., 2009). Brown seaweed polysaccharides including laminarin (β-glucan) and fucoidan (a sulphated polysaccharide) exhibit bioactivities such as anti-adhesive, anticoagulant, anti-inflammatory, antioxidant and antitumoral properties (Eluvakkal et al., 2010). Antioxidant activity of laminarin and fucoidan depends on several structural parameters, such as the type of sugar and glycosidic branching, molecular weight and the degree and position of sulphation (Jiménez-Escrig et al., 2012). Laminarin contains two types of polymeric chains, one where glucose is attached to the end of the chain (G-chain) and the other has mannitol as the terminal reducing end (M-chain) (Choi et al., 2011). Fucoidan extracted from *Laminaria digitata* is reported to contain fucose and sulphates as well as xylose, mannose, glucose, galactose and uronic acid in minor amounts (Li et al., 2008). Understanding the mechanism (inhibit/scavenge reactive species, prevent/terminate free-radical generating reactions or chelate/sequester metals) through which compounds exert their antioxidant activity can help determine the mode of

action/efficacy of antioxidant compounds in muscle, following digestion (Decker, et al., 2000).

Consumer concerns over the safety and toxicity of synthetic antioxidants in meat products has lead to increased research into the use of natural antioxidant compounds. The perceived healthiness and structure of seaweed make it an ideal source of bioactive compounds with antioxidant activities which may replace synthetic antioxidants in meat products. In Chapter 4, the brown seaweed extract, containing laminarin and fucoidan, decreased lipid oxidation in fresh pork LTL. Pigs from the same study displayed improved gut health due to laminarin and fucoidan from the dietary seaweed extract (Murphy et al., 2013). However, further investigation is necessary to determine the level and duration of feeding dietary seaweed extracts containing laminarin and fucoidan in order to optimise fresh pork quality and shelf-life.

The objective of this study was to examine the effects of supplementation of porcine diets with a polysaccharide (laminarin (L) and fucoidan (F)) (L/F) based seaweed extract at two levels (450 or 900 mg/kg feed) and durations (3 or 6 wks) of feeding, on the quality, shelf-life parameters and sensory properties of fresh *longissimus thoracis et lumborum* (LTL) steaks. The influence of dietary L/F on iron-induced lipid oxidation in porcine liver, heart, kidney and lung tissue homogenates was assessed. The mechanism of action of dietary L/F on the stability of lipids in pork meat was also investigated.

5.2 MATERIALS AND METHODS

5.2.1 Reagents

All chemicals used were 'AnalaR' grade obtained from Sigma-Aldrich Ireland Ltd., Arklow, Co. Wicklow, Ireland, Oxoid Ltd., Basingstoke, Hampshire, England, Fisher Scientific, Dublin, Ireland and Merck KGaA, Darmstadt, Germany. The total antioxidant status (TAS) Randox-Trolox kit was obtained from Randox Laboratories Ltd., Co. Antrim, UK. A wet formulation seaweed extract (L/F), containing laminarin (L) and fucoidan (F) was manufactured by Bioatlantis, Tralee, Co. Kerry, Ireland. The extract isolated from brown seaweed (*Laminaria digitata*), harvested in Ireland, was prepared using an acid extraction technique, details of which are industrially-confidential. The composition of L/F is outlined in Table 5.1. The ash content was supplied by the manufacturer.

Table 5.1. Composition of the seaweed extract containing laminarin (L) and fucoidan (F) (L/F).

	L/F extract
% Total Solids	38.9
% Ash[a]	28.1
% Protein	0.25
% Laminarin	4.0
% Fucoidan	3.2
% Mannitol	3.3
Inclusion rate (per tonne feed) (L/F_{450})	6.25 Kg
Inclusion rate (per tonne feed) (L/F_{900})	12.5 Kg

[a]The ash content of the seaweed extract was 15 g/kg DM Ca, 10 g/kg DM Na, 10 g/kg DM K, 10 g/kg DM S, 250 mg/kg DM iodine, 250 mg/kg DM Fe, 20 mg/kg DM Cu and 50 mg/kg DM Zn.

5.2.2 Animals and diets

Thirty pigs (Large White x Landrace crosses consisting of 15 males and 15 females) (average live weight ~ 82 kg, age: 16 weeks) were randomly assigned to one of five dietary treatments (n = 6) and fed *ad libitum* a basal diet plus the L/F extract for 3 or

6 weeks pre-slaughter, following a completely randomised experimental design. The control group were fed the basal diet for the duration of the experiment. The composition and analysis of the basal diet is reported in Chapter 4. The seaweed extract in the feed formulation was added to result in total L and F concentrations of 450 or 900 mg/kg feed. Average daily feed intake, average daily gain, feed conversion ratio and details of the dietary treatments of each group are outlined in Table 5.2.

Table 5.2. Average daily feed intake (ADF) (kg/day), Average daily gain (ADG) (kg/day), Feed conversion ratio (FCR), level (mg/kg feed) and duration (weeks) of dietary L/F.

	ADF[x]	**ADG[y]**	**FCR[z]**	**L level**	**F level**	**Total L/F level**	**Duration**
Control[w]	2.13	0.810	2.62	--	--	--	6
L/F_{450-3}	2.20	0.805	2.68	250	200	450	3
L/F_{450-6}	2.18	0.812	2.68	250	200	450	6
L/F_{900-3}	2.15	0.820	2.62	500	400	900	3
L/F_{900-6}	2.13	0.810	2.63	500	400	900	6

[w]Control group fed basal diet only
[x]SEM 0.05
[y]SEM 0.03
[z]SEM 0.07

The animals were housed individually (1.68 m × 1.22 m slatted pens) at Lyons Research Farm, University College Dublin, Newcastle, Co. Dublin, Ireland and fed *ad libitum* from hopper-style feeders. Water was supplied *ad libitum* from individual nipple drinkers. The ambient environmental temperature within the houses was thermostatically controlled and maintained at 22°C. Blood samples for plasma analysis were taken prior to transportation to the abattoir (Rosderra Irish Meats Group, Edenderry, Co. Offaly, Ireland). Pigs were stunned using gas and humanely slaughtered, 3 hr after feeding, following animal welfare regulations. The mean slaughter weight was 115.81 kg. The liver, heart, kidney and lungs were removed from each animal, placed in laminate vacuum pack bags (composed of polyamide/polyethylene layers), vacuum packed and stored at -18ºC for one month prior to analysis. Blood samples were centrifuged (Beckman J2-21, Beckman Instruments Inc., CA, USA) at 4720g for 20 min

at 4ºC, and plasma fractions were removed and stored at -20ºC prior to analysis. Carcasses were hung at 2ºC for 24 hr at Rosderra Irish Meats Group then transported at 4ºC to the School of Food and Nutritional Sciences at University College Cork, Ireland and stored at 2ºC for a further 24 hr before removal of muscles for analysis.

5.2.3 Plasma total antioxidant status

The total antioxidant status (TAS) of porcine blood plasma was measured as described in Chapter 4 (section 4.2.3) and the results were expressed as mmol of trolox equivalent antioxidant capacity (TEAC)/L plasma.

5.2.4 Pork processing and packaging

Longissimus thoracis et lumborum (LTL) muscles were excised from each carcass, vacuum packed and stored at 2ºC for 24 hr. LTL were cut into steaks (~ 2.5cm in thickness, ~ 45 g portion), placed in low oxygen permeable (<1cm^3/m^2/24 h at STP) polystyrene/ethylvinylalcohol (EVOH)/polyethylene (PE) trays. Trays were covered using a low oxygen permeable (3 cm^3/m^2/24 h at STP) laminated barrier film with a polyolefin heat-sealable layer. Trays were flushed with 80% O_2 : 20% CO_2 (modified atmosphere packs, MAP) using a vacuum-sealing unit (VS 100, Gustav Müller & Co. KG, Homburg, Germany) equipped with a gas mixer (Witt-Gasetechnik GmbH & Co. KG, Witten, Germany) and heat-sealed. LTL steaks in MAP were stored for up to 14 days under fluorescent lighting (660 lux) at 4ºC. The gas atmosphere (% O_2 and % CO_2) in the MAP was checked using a CheckMate 9900 (PBI-DanSensor, Denmark). Immediately after gas flushing, MAP contained 75.07 ± 0.43% O_2 and 25.62 ± 0.29% CO_2. The average gas composition in MAP at the end of the 14 day storage period was 73.01 ± 0.88% O_2 and 26.83 ± 0.87% CO_2.

In the cooked pork study, LTL muscles from all treatments were trimmed of visible fat and connective tissue and minced twice through a plate with 4 mm holes (Model P114L, Talsa, Valencia, Spain), formed into patties (100 g portions) using a meat former (Ministeak burger maker, O.L Smith Co. Ltd., Italy), placed on aluminium foil lined trays and cooked at 180°C for 20 min in a fan-assisted convection oven (Zanussi Professional, Model 10 GN1/1, Conegliano, Italy) until an internal temperature of 72°C was reached. Cooked patties were placed either in trays and either flushed with 70% N_2: 30% CO_2 (MAP) and stored for up to 14 days as described previously, or over-wrapped with oxygen permeable low-density polyethylene film and stored aerobically for up to 3 days at 4°C (approximately 660 lx). Immediately after gas flushing, MAP contained 67.55 ± 0.93% N_2 and 32.25 ± 0.72% CO_2. The average gas composition in MAP at the end of the 14-day storage period was 67.89 ± 0.16% N_2 and 30.98 ± 0.06% CO_2. Percent nitrogen was calculated by difference of % O_2 and % CO_2 (CheckMate 9900 (PBI-DanSensor, Denmark)).

5.2.5 Measurement of pork quality and shelf-life parameters

Pork pH, colour (CIE 'L*' lightness, 'a*' redness and 'b*' yellowness values), lipid oxidation (2-thiobarbituric acid reactive substances (TBARS)), microbiological analysis (mesophilic and psychrotrophic total viable counts), and sensory evaluation ('visual' and 'eating quality') were measured at intervals during storage as described in Chapter 4 (sections 4.2.5-4.2.9). The pH, colour measurements and lipid oxidation in fresh LTL steaks and cooked minced pork patties were recorded on days 1, 4, 7, 11 and 14 of storage. In cooked minced pork patties stored aerobically, lipid oxidation was measured on days 0, 1, 2 and 3 of storage. Microbiological analysis of fresh LTL steaks was carried out on days 1, 5, 8 and 13 of storage. Sensory analysis ('visual' and 'eating

quality') of fresh LTL steaks stored in 80% O_2 : 20% CO_2 was performed in duplicate by 52 naïve assessors on days 1 and 7 of storage. 'Visual' sensory analysis descriptors of fresh LTL steaks were pinkness, whiteness, drip, package quality, purchasing appeal, and overall acceptability. 'Eating quality' sensory analysis descriptors of cooked LTL steaks (180°C for 20 min in a fan-assisted convection oven) were appearance, tenderness, oxidation flavour, liking of flavour and overall acceptability.

5.2.6 The mechanism of action of L/F on lipid stability in pork meat

5.2.6.1 Fatty acid analysis

Lipids were extracted from pork samples with chloroform : methanol (2:1 v/v) according to the method by Folch et al. (1957). Fatty acid methyl esters (FAMEs) were prepared by first using 10 ml 0.5N NaOH in methanol for 10 min at 90°C followed by 10 ml 14% BF_3 in methanol for 10 min at 90°C (Park & Goins, 1994). FAMEs were recovered with hexane. Prior to gas chromatography (GC) analysis, samples were dried over anhydrous sodium sulphate (0.5g) for 1 hr and stored at -20°C. FAMEs were separated using GC (Varian 3800, Varian, Walnut Creek, CA, USA) fitted with a flame ionization detector, using a Chrompack CP Sil 88 column (Chrompack, Middleton, The Netherlands, 100 m × 0.25 mm i.d., 0.20 μm film thickness) and helium as the carrier gas. The column oven was programmed to be held initially at 80°C for 8 min and increased 8.5°C/min to a final column temperature of 200°C. The injection volume used was 0.6 μl, with automatic sample injection on a SPI 1093 splitless on-column temperature programmable injector. Peaks were integrated using the Varian Star Chromatography Workstation software (version 6.0) and peaks were identified by comparison of retention times with pure FAME standards (Nu-Chek Prep, Elysian, MN, USA). The percentage of

individual fatty acids was calculated according to the peak areas relative to the total area (total fatty acids were set at 100%). Results were expressed as g/100g FAME.

5.2.6.2 *In vitro* antioxidant activity of L/F in pork meat

Pork homogenates (10% w/v) were prepared as described by Qwele, et al. (2013) for measurement of *in vitro* antioxidant activity. Briefly, LTL steaks (10 g) were homogenised in 0.05 M phosphate buffer (90 ml), pH 7, using an Ultra Turrax T25 homogeniser. Trolox C (1000 µg/ml), EDTA (1000 µg/ml) were added to 10% pork homogenates as positive controls for the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging and the ferrous ion chelating activity (FICA) assays. A spray-dried seaweed extract containing 9.3% L and 7.8% F, comparable to the wet extract used in the animal diets, was added to 10% pork homogentates (1000 and 3000 µg/ml) as a positive seaweed polysaccharide control for both DPPH and FICA assays. Homogenates were centrifuged at 7,800g for 10 min at 4°C. The supernatants obtained were used for the estimation of the DPPH free radical scavenging and FICA activities of L/F in pork meat.

5.2.6.2.1 Determination of DPPH radical scavenging activity

DPPH radical-scavenging activity of LTL steaks was measured as described by Qwele, et al., (2013) with slight modifications. DPPH (0.2 mM, 3 ml) in methanol was added to 0.3 ml supernatant and 2.7 ml distilled water. The mixture was vortexed and left to stand at room temperature (20–22°C) in the dark and the precipitate formed was removed by filtering through Whatman No. 1 filter paper. The assay control contained 0.3 ml buffer (0.05 M phosphate), 2.7 ml distilled water and 3 ml of DPPH solution. Absorbances were measured against a methanol blank after 20 hr at 517 nm (Cary 300 Bio, UV-Vis spectrophotometer, Varian Instruments, CA, USA). Trolox C (1000 µg/ml)

and L/F (1000 and 3000 μg/ml) were used as positive controls. The DPPH radical scavenging activity expressed as percent of the control was calculated as follows:

% inhibition of DPPH = [1− (absorbance of sample / absorbance of control)] x 100

5.2.6.2.2 Determination of ferrous ion chelating activity (FICA)

The FICA of L/F in pork meat was measured using the iron-ferrozine complex method (Yen & Wu, 1999) with slight modifications. $FeCl_2$ (2 mM, 0.1 ml) in distilled water was added to 0.5 ml supernatant and 4.2 ml distilled water. The reaction was initiated with the addition of 0.2 ml of 5 mM ferrozine in distilled water, the mixture was vortexed and left to stand at room temperature (20–22°C) for 1 hr in the dark. The assay control contained 0.5 ml buffer (0.05 M phosphate), 4.2 ml distilled water, 0.1 ml of $FeCl_2$ and 0.2 ml of ferrozine solution. At 1 hr the absorbance of the solution was measured at 562 nm against a water blank using a spectrophotometer (Cary 300 Bio). EDTA (1000 μg/ml) and L/F (1000 and 3000 μg/ml) were used as positive controls. The FICA was calculated as follows:

% chelating activity = [1− (absorbance of sample / absorbance of control)] x 100

5.2.7 Statistical analysis

All analyses were performed in duplicate. Mean sample values (n = 6) for each of the five treatment groups (Control, $L/F_{450\ -\ 3}$, $L/F_{450\ -\ 6}$, $L/F_{900\ -\ 3}$ and $L/F_{900\ -\ 6}$) were subjected to statistical analysis. A full repeated measures two-way analysis of variance (ANOVA) was conducted to investigate the effects of dietary L/F level (450 or 900), duration (3 wk or 6 wk) and level*duration interactions. No significance differences were observed between level*duration interactions. Level and duration represented the 'between-subjects' factor and the effect of time was measured using the 'within-subjects'

factor. Tukey's test was used to adjust for multiple comparisons between treatment means. The analysis was performed using the SPSS 18.0 for Windows (SPSS, Chicago, IL, USA) software package.

'Visual' and 'eating quality' sensory data was analysed with ANOVA-Partial Least Squares Regression (APLSR) to process the mean data accumulated from the 52 test subjects. 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. In these models assessor and session level effects were removed using level correction. The validated model explained variance was 13.8% on day 1 and 18.0% on day 7 and the calibrated variance was 17.5% on day 2 and 21.4% on day 7. 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 the Unscrambler Software, version 9.8 (CAMO ASA, Trondheim, Norway).

5.3 RESULTS AND DISCUSSION

5.3.1 Plasma total antioxidant status

The TAS of porcine plasma was 1.03, 1.01, 0.91, 1.04 and 0.99 mmol TEAC/L plasma for the control, L/F_{450-3}, L/F_{450-6}, L/F_{900-3} and L/F_{900-6}, groups respectively. Results indicated that level of L/F dietary addition or duration of feeding did not influence ($p > 0.05$) the TAS of porcine plasma. Similar findings were reported in Chapter 4 where porcine plasma TAS was not affected by L/F supplementation at a level and duration similar to L/F_{900-3}. Similarly, dried *Ascophyllum nodosum* (2.5, 5.0 or 10.0 g / kg) did not affect a range of markers for plasma oxidative status, including MDA, after inclusion in pigs diets for 28 days (Michiels et al., 2011). In the present study, pigs were fed 3 hours pre-slaughter and lack of an effect of L/F on porcine plasma TAS may have been due to the rapid absorption during digestion.

5.3.2 Colour stability and muscle pH of fresh LTL muscle

The surface lightness '*L**' increased ($p < 0.05$) in fresh LTL muscle over the 14-day storage period. Trends indicated surface yellowness 'b*' values increased ($p > 0.05$) over time, however results were not statistically significant. The '*a**' redness values of LTL muscle decreased ($p < 0.05$) as a function of storage time and was not influenced by level or duration of feeding dietary L/F (Table 5.3). Similar trends in the colour stability of pork meat were reported in Chapter 4. In addition, Park et al. (2005) demonstrated that supplementation of pig diets with a polysaccharide iron complex (chitosan-alginate-Fe(II) (3 ml per day)) had no effect on LTL pork steak colour parameters.

Muscle pH of fresh pork ranged from 5.61 to 5.15 over the 15-day storage period and was unaffected by level and duration of feeding dietary L/F. The pH values reported

Table 5.3. Effect of dietary laminarin/fucoidan (L/F) on the surface lightness ('*L**' value), redness ('*a**' value) and yellowness ('*b**' value) values of fresh *longissimus thoracis et lumborum* (LTL) stored in modified atmosphere packs (80% O_2 : 20% CO_2) for up to 14 days at 4°C.

Treatment	Parameter	Storage time at 4°C, days				
		1	**4**	**7**	**11**	**14**
Control	Lightness	54.99 ± 2.83^{aA}	55.02 ± 2.32^{aA}	55.74 ± 2.14^{aAB}	56.38 ± 2.07^{aAB}	59.01 ± 2.06^{aB}
L/F_{450-3}	*L**	54.42 ± 4.09^{aA}	54.73 ± 5.01^{aA}	57.14 ± 3.96^{aA}	57.59 ± 4.40^{aA}	59.11 ± 3.53^{aA}
L/F_{450-6}		53.21 ± 2.47^{aA}	54.23 ± 2.59^{aAB}	56.17 ± 2.09^{aABC}	57.60 ± 1.28^{aBC}	58.85 ± 1.60^{aC}
L/F_{900-3}		52.94 ± 2.77^{aA}	54.50 ± 2.41^{aAB}	55.62 ± 1.55^{aAB}	56.01 ± 0.93^{AB}	56.82 ± 1.45^{aC}
L/F_{900-6}		54.54 ± 2.30^{aA}	56.11 ± 1.68^{aAB}	58.17 ± 3.35^{aAB}	56.85 ± 2.46^{aAB}	58.99 ± 1.80^{aC}
Control	Redness	6.75 ± 1.12^{aA}	6.45 ± 0.41^{aA}	5.53 ± 0.57^{aAB}	4.13 ± 0.72^{aBC}	3.73 ± 1.51^{aC}
L/F_{450-3}	*a**	6.80 ± 0.62^{aA}	6.64 ± 0.55^{aA}	5.18 ± 0.53^{aB}	5.14 ± 0.78^{aB}	4.18 ± 1.10^{aB}
L/F_{450-6}		6.19 ± 1.01^{aA}	5.79 ± 0.53^{aAB}	5.34 ± 1.04^{aAB}	4.77 ± 0.72^{aBC}	3.49 ± 0.76^{aC}
L/F_{900-3}		5.97 ± 0.74^{aA}	5.20 ± 0.76^{aA}	4.97 ± 0.65^{aAB}	3.92 ± 0.56^{aC}	3.89 ± 0.43^{aC}
L/F_{900-6}		6.39 ± 1.21^{aA}	5.49 ± 1.39^{aAB}	4.79 ± 1.20^{aABC}	4.00 ± 0.93^{aBC}	3.29 ± 1.27^{aC}
Control	Yellowness	7.99 ± 0.88^{aA}	8.09 ± 0.44^{aA}	7.84 ± 0.51^{aA}	7.42 ± 0.46^{aA}	8.67 ± 1.55^{aA}
L/F_{450-3}	*b**	8.26 ± 0.96^{aA}	8.35 ± 1.11^{aA}	8.34 ± 1.04^{aA}	8.85 ± 1.03^{aA}	9.04 ± 1.43^{aA}
L/F_{450-6}		7.46 ± 0.81^{aA}	7.29 ± 0.39^{aA}	7.81 ± 0.57^{aA}	8.29 ± 0.82^{aA}	7.95 ± 1.20^{aA}
L/F_{900-3}		7.26 ± 0.58^{aA}	7.22 ± 0.42^{aA}	7.60 ± 0.70^{aA}	7.35 ± 0.35^{aA}	7.73 ± 0.46^{aA}
L/F_{900-6}		7.48 ± 0.75^{aA}	7.51 ± 0.83^{aA}	7.89 ± 1.29^{aA}	7.39 ± 0.98^{aA}	7.99 ± 1.06^{aA}

[a]Within each parameter and storage day, mean values (± standard deviation) in the same column are not significantly different, $p > 0.05$.
[ABC]Within each treatment, mean values (± standard deviation) in the same row bearing different superscripts are significantly different, $p < 0.05$.

were comparable to previously reported values (5.90-5.19) for post-mortem pork muscle from pigs fed L/F in Chapter 4. The relationship between muscle pH and pork colour (*L** and *a**) has been established where pH-induced effects on muscle proteins directly correlated with changes in the pink colour of pork, as observed in pale soft exudative (PSE) and dark firm dry pork meat (DFD) (Brewer et al., 2001). In the present study, no effect was exerted by level or duration of feeding L/F on muscle pH or the colour stability of fresh pork LTL steaks.

5.3.3 Lipid oxidation in fresh LTL muscle and cooked minced pork

Lipid oxidation significantly ($p < 0.05$) increased in meat from all dietary groups as a function of storage time (Table 5.4). However, the levels of lipid oxidation were generally low with mean values ranging from 0.16-0.93 mg MDA/kg pork. Trends for lipid oxidation followed the order: $C = L/F_{450-6} = L/F_{900-6} > L/F_{450-3} = L/F_{900-3}$. On day 11 and 14 of storage in MAP, levels of lipid oxidation in L/F_{450-3} and L/F_{900-3} were significantly ($p < 0.05$) lower compared to the control. In Chapter 4, protection against lipid oxidation was reported in LTL steaks potentially due to deposition of antioxidant components in L/F in pork muscle following dietary supplementation at a level similar to L/F_{900-3}.

Recent studies have shown that dietary L/F modulated gastrointestinal physiology, improved digestion and increased absorptive functions of the pig intestine (Heim et al., 2014). The degree of digestibility of the seaweed polysaccharides increased with feeding duration, which subsequently increased nutrient digestibility (O'Doherty et al., 2010). In the present study, L/F_{450-3} and L/F_{900-3} was sufficient to increase lipid stability in LTL steaks. Uptake and increased utilisation of the polysaccharides by gut microbes may be

Table 5.4. Effect of dietary laminarin/fucoidan (L/F) on lipid oxidation (TBARS*) in fresh *longissimus thoracis et lumborum* (LTL) and cooked pork patties stored in modified atmosphere packs (MAP) and aerobically for up to 14 days and 3 days, respectively, at 4°C.

Treatment	**Packaging**		**Storage time at 4°C, days**				
			1	**4**	**7**	**11**	**14**
Control	MAP[1]	Fresh	0.16 ± 0.01^{aA}	0.17 ± 0.03^{aA}	0.20 ± 0.04^{aA}	0.60 ± 0.26^{aB}	0.93 ± 0.33^{aC}
L/F_{450-3}			0.16 ± 0.02^{aA}	0.19 ± 0.04^{aAB}	0.17 ± 0.06^{aA}	0.32 ± 0.08^{bBC}	0.40 ± 0.16^{bC}
L/F_{450-6}			0.16 ± 0.02^{aA}	0.17 ± 0.03^{aA}	0.17 ± 0.04^{aA}	0.45 ± 0.21^{abAB}	0.65 ± 0.51^{abB}
L/F_{900-3}			0.16 ± 0.02^{aA}	0.16 ± 0.02^{aA}	0.16 ± 0.02^{aA}	0.31 ± 0.10^{bB}	0.37 ± 0.13^{bB}
L/F_{900-6}			0.16 ± 0.01^{aA}	0.16 ± 0.03^{aA}	0.18 ± 0.03^{aA}	0.35 ± 0.09^{abAB}	0.52 ± 0.26^{abB}
Control	MAP[2]	Cooked	1.78 ± 0.42^{aA}	2.91 ± 0.60^{aB}	2.90 ± 0.55^{aB}	3.07 ± 0.60^{aB}	2.89 ± 0.45^{aB}
L/F_{450-3}			1.76 ± 0.49^{aA}	2.66 ± 0.62^{aA}	2.39 ± 0.48^{aA}	2.63 ± 0.68^{aA}	2.54 ± 0.57^{aA}
L/F_{450-6}			2.03 ± 0.36^{aA}	3.05 ± 1.17^{aA}	3.12 ± 0.71^{aA}	2.96 ± 0.47^{aA}	3.02 ± 0.59^{aA}
L/F_{900-3}			1.71 ± 0.18^{aA}	2.28 ± 0.19^{aB}	2.47 ± 0.29^{aB}	2.65 ± 0.45^{aB}	2.55 ± 0.36^{aB}
L/F_{900-6}			2.11 ± 0.50^{aA}	3.04 ± 0.94^{aAB}	3.08 ± 0.57^{aAB}	3.35 ± 0.81^{aB}	2.65 ± 0.48^{aAB}
			0	1	2	3	
Control	Aerobic	Cooked	1.21 ± 0.34^{aA}	2.03 ± 0.54^{aAB}	2.59 ± 0.53^{aBC}	3.39 ± 0.88^{aC}	
L/F_{450-3}			1.26 ± 0.18^{aA}	1.85 ± 0.58^{aA}	2.32 ± 0.77^{aAB}	2.94 ± 1.02^{aC}	
L/F_{450-6}			1.08 ± 0.29^{aA}	2.34 ± 0.51^{aB}	3.13 ± 0.74^{aBC}	3.75 ± 0.76^{aC}	
L/F_{900-3}			1.09 ± 0.14^{aA}	1.81 ± 0.22^{aB}	2.40 ± 0.42^{aC}	3.11 ± 0.54^{aD}	
L/F_{900-6}			1.40 ± 0.19^{aA}	2.51 ± 0.54^{aB}	2.92 ± 0.34^{aBC}	3.48 ± 0.35^{aC}	

[1](80% O_2 : 20% CO_2), [2](70% N_2 : 30% CO_2). *TBARS, mg malondialdehyde (MDA)/kg pork.

[ab]Within each parameter and storage day, mean values (± standard deviation) in the same column bearing different superscripts are significantly different, $p < 0.05$.

[ABCD]Within each treatment, mean values (± standard deviation) in the same row bearing different superscripts are significantly different, $p < 0.05$.

responsible for the differences observed in lipid stability of L/F_{450-6} and L/F_{900-6} compared to feeding L/F for 3 weeks.

In cooked pork patties stored in MAP, trends indicated that lipid oxidation increased in all treatments over the 14-day storage period, however results were only statistically significant ($p < 0.05$) for the control and L/F_{900-3}. In cooked pork patties stored aerobically, lipid oxidation increased significantly ($p < 0.05$) in all treatments over the 14-day storage period (Table 5.4). Lowest levels of lipid oxidation for cooked pork patties stored aerobically and in MAP were observed in L/F_{450-3} and L/F_{900-3} and results were not significantly different from the controls. Lipid oxidation levels were greater than those observed for raw LTL muscle due to the pro-oxidative nature of the mincing and cooking processes. In cooked patties stored in MAP, lipid oxidation was lower than in patties stored aerobically due to the low oxygen levels present in MAP. In the present study, although not statistically significant, trends showed that dietary L/F supplementation for 3 weeks resulted in slightly lower levels of lipid oxidation compared to the controls for cooked patties stored in both packaging conditions. The behaviour of dietary seaweed polysaccharides during cooking requires further investigation.

5.3.4 Lipid oxidation in porcine liver, heart, kidney and lung tissue homogenates

Liver, heart, kidney and lung tissue homogenates were subjected to iron ($FeSO_4$)-induced lipid oxidation to estimate the distribution of L/F extract in porcine organ tissues. Following $FeSO_4$ addition, lipid oxidation increased in all tissue homogenates over the 24 hr storage period (Table 5.5). In Chapter 4, slightly lower levels of lipid oxidation were observed in porcine liver tissue homogenates after dietary supplementation of a spray-dried seaweed extract similar to L/F_{900-3}. In the present study, similar decreases in levels of lipid oxidation of liver tissues after L/F supplementation were not detected. Lynch et

al. (2010) suggested that pig maturity influenced the digestion, breakdown and subsequent availability of fermentable polysaccharides entering the large intestine. Pigs used in this chapter (115.81 kg) were larger in size than pigs (29.7 kg) investigated in Chapter 4. The differences in lipid stability of organ tissues observed between the two studies may be attributed to maturity of the pigs at the time of slaughter.

Table 5.5. Effect of dietary laminarin/fucoidan (L/F) on iron-induced lipid oxidation (TBARS) in organ (liver, heart, kidney and lung) tissue homogenates after 24 hours storage at 4ºC.

Treatment	**Storage for 24 hours at 4ºC**			
	liver	**heart**	**kidney**	**lung**
Control	2.91 ± 0.76[a]	1.42 ± 0.88[a]	2.68 ± 0.82[a]	1.26 ± 0.55[a]
L/F_{450-3}	2.93 ± 0.81[a]	1.21 ± 0.70[a]	2.52 ± 0.46[a]	1.21 ± 0.71[a]
L/F_{450-6}	3.21 ± 0.85[a]	1.76 ± 0.81[a]	2.72 ± 0.55[a]	1.55 ± 0.77[a]
L/F_{900-3}	3.10 ± 0.67[a]	1.52 ± 0.31[a]	1.72 ± 0.90[a]	1.20 ± 0.65[a]
L/F_{900-6}	2.98 ± 0.39[a]	1.17 ± 0.35[a]	1.59 ± 0.65[a]	0.86 ± 0.14[a]

[a]Within each organ type, mean values (± standard deviation) in the same column are not significantly different, $p > 0.05$.
*TBARS, mg malondialdehyde (MDA)/kg organ tissue.

Several studies have shown that dietary seaweed extracts, containing laminarin and fucoidan individually, can accumulate and positively influence stress markers in animal liver, heart, kidney and lung tissues (Thomes, et al., 2010). In rat lung tissue, deposition of antioxidant components from dietary laminarin (200 or 400 mg/kg body weight) was found to significantly reduce malondialdehyde (MDA) levels (Cheng et al., 2011). In the present study, the maximum level and duration (L/F_{900-6}) was the most effective (not significantly) of all treatments examined at reducing iron-induced oxidation of porcine kidney and lung tissue homogenates compared to the control. The digestion of soluble dietary fibre has been linked to changes in microbiota which consequently influences absorption rate of minerals and other nutrients (Scholz-Ahrens et al., 2001). The digestion and utilisation of dietary L/F by the porcine gut to support immunological health may have influenced the bioavailability and subsequent deposition of L/F and minerals from the extract in the organ tissues. Increased availability of iron and copper,

known initiators of lipid oxidation, from the seaweed extract may have counterbalanced the antioxidant potential of dietary L/F.

5.3.5 Microbiology of fresh LTL muscle

The mesophilic plate counts of fresh LTL muscle ranged from ~2.7 to ~3.2 log cfu/g on day 1 and increased to a maximum of ~7.0 log cfu/g on day 13 of storage (Table 5.6). Psychrotrophic plate counts ranged from ~4.0 to ~5.0 log cfu/g on day 1 and increased to levels ranging from ~9.9 to ~10.5 log cfu/g on day 13 (Table 5.6). Mesophilic and psychrotrophic counts are in agreement with previously reported values from Chapter 4. Results from the present study indicated dietary L/F, regardless of level or duration of feeding, did not exert antimicrobial activity in fresh LTL steaks.

The biological activity of sulphated polysaccharides can vary between species and has been linked to the molecular weight and sulphated content as well as the position of sulphate groups (Li, et al., 2008). Antimicrobial properties of seaweed extracts are influenced by extraction solvents, form and concentration of compounds present, and activity has been reported in extracts prepared from a range of seaweed species (Bansemir et al., 2006; Cox et al., 2013). Crude extracts containing sulphated polysaccharides from *Gracilaria ornata* exhibited no antimicrobial activity against seven bacteria (*B. subtilis*, *S. aureus*, *E. aerogens*, *E. coli*, *P. aeruginosa*, *S. choleraesuis* and *S. typhi*) (plate diffusion method). Amorim et al. (2012) postulated the absence of antimicrobial activity of sulphated polysaccharides may be due to the inability to interact with the cell wall of bacteria as a result of charge and the repulsion of the sulphated groups. In the present study, the lack of antimicrobial activity in the LTL steaks may be attributed to the form and concentration of compounds deposited in the muscle.

Table 5.6. Effect of dietary laminarin/fucoidan (L/F) on the microbial status* (mesophilic and psychrotrophic TVC) of fresh *longissimus thoracis et lumborum* (LTL) stored in modified atmosphere packs (80% O_2 : 20% CO_2) at for up to 13 days at 4°C.

Treatment		**Storage time at 4°C, days**			
	Temp	**1**	**5**	**8**	**13**
Control	30°C	2.84 ± 0.27^{aA}	3.78 ± 0.01^{aB}	4.83 ± 0.22^{aC}	6.58 ± 0.39^{aCD}
L/F_{450-3}	mesophilic	2.77 ± 0.20^{aA}	4.03 ± 0.05^{aAB}	5.14 ± 0.49^{aB}	5.46 ± 0.92^{aC}
L/F_{450-6}	count	3.16 ± 0.08^{aA}	3.76 ± 0.19^{aA}	6.14 ± 0.73^{aB}	6.83 ± 0.84^{aC}
L/F_{900-3}		2.66 ± 0.26^{aA}	3.78 ± 0.02^{aA}	5.20 ± 0.41^{aB}	7.01 ± 0.53^{aB}
L/F_{900-6}		2.70 ± 0.30^{aA}	3.65 ± 0.25^{aAB}	4.73 ± 0.55^{aB}	6.49 ± 0.55^{aB}
Control	4°C	4.42 ± 0.40^{aA}	4.80 ± 0.43^{aAB}	6.49 ± 0.83^{aB}	9.90 ± 0.73^{aC}
L/F_{450-3}	psychrotrophic	4.68 ± 0.77^{aA}	4.92 ± 0.74^{aA}	6.54 ± 0.31^{aB}	10.17 ± 0.20^{aC}
L/F_{450-6}	count	4.95 ± 0.28^{aA}	5.69 ± 0.29^{aB}	7.24 ± 0.43^{aC}	10.50 ± 0.07^{aD}
L/F_{900-3}		4.00 ± 0.42^{aA}	5.18 ± 0.21^{aA}	6.37 ± 0.37^{aB}	10.49 ± 0.02^{aC}
L/F_{900-6}		4.12 ± 0.18^{aA}	4.65 ± 0.30^{aAB}	6.77 ± 0.09^{aB}	10.29 ± 0.04^{aC}

[a]Within each storage temperature and day, mean values (± standard deviation) in the same column are not significantly different, $p > 0.05$.

[ABCD]Within each treatment, mean values (± standard deviation) in the same row bearing different superscripts are significantly different, $p < 0.05$.

*$\log_{10}$CFU (colony forming units)/g pork.

5.3.6 Sensory evaluation of fresh and cooked LTL muscle

In 'visual' sensory analysis of LTL steaks (Table 5.7), no significant trends were observed for 'pinkness' or 'whiteness' with the exception of L/F_{450-3} which was significantly ($p < 0.05$) positively correlated to appearing more (pink) on day 1. Instrumental '*a**' redness values support such findings indicating that colour was unaffected by level and duration of feeding dietary L/F. On day 1, L/F_{900-3} was very highly significantly ($p < 0.001$) positively correlated to having a high drip loss while L/F_{900-6} was highly significantly ($p < 0.01$) negatively correlated to having no drip loss. On day 7, the control was very highly significantly ($p < 0.001$) positively correlated to having high drip loss while L/F_{450-3} was very highly significantly ($p < 0.001$) negatively correlated to a lack of drip loss. Although not significant, L/F_{450-6}, L/F_{900-3}, L/F_{900-6} were also negatively correlated to having a lack of drip loss on day 7, which may indicate that deposition of components of the L/F extract led to an increase in stability in the protein matrix leading to reduced drip during storage. On day 7, L/F_{450-3} was significantly ($p < 0.05$) positively correlated to purchasing appeal and overall visual acceptability while the control was significantly ($p < 0.05$) negatively correlated to purchasing appeal and overall visual acceptability. Therefore, the 'visual' sensory properties of L/F_{450-3} pork patties were enhanced by dietary L/F supplementation according to the sensory panellists.

No significant trends were observed for 'eating quality' sensory analysis including appearance, texture, and overall acceptability of the cooked LTL muscle (Table 5.7). In a previously reported study, the addition of dried *Himanthalia elongata* (5%) to low-fat frankfurters resulted in less acceptable products, due mainly to the flavour of the seaweed (López-López et al., 2010). In the present study, 'eating quality' sensory analysis was

Table 5.7. Significance of regression coefficients (ANOVA values) for fresh *longissimus thoracis et lumborum* (LTL) as derived by jack-knife uncertainty testing.

Parameter	**Storage time, days / Treatment**									
	Control		**L/F$_{450 - 3}$**		**L/F$_{450 – 6}$**		**L/F$_{900 - 3}$**		**L/F$_{900 - 6}$**	
Visual Sensory Analysis	**1**	**7**	**1**	**7**	**1**	**7**	**1**	**7**	**1**	**7**
Pinkness	0.61	-0.40	0.01*	0.45	0.80	0.93	-0.31	0.48	-0.47	0.55
Whiteness	-0.64	-0.99	-0.07	0.99	-0.81	0.99	0.39	0.99	0.52	0.99
Drip	0.27	0.00***	0.23	-0.00***	0.72	-0.93	0.00***	-0.23	-0.01**	-0.57
Packaging quality	-0.67	0.99	-0.66	-0.99	-0.78	-0.99	0.65	-0.99	0.64	-0.99
Purchasing appeal	-0.69	-0.03*	-0.79	0.05*	-0.56	0.93	0.75	0.29	0.73	0.56
Overall acceptability	-0.41	-0.03*	-0.68	0.05*	-0.51	0.93	0.59	0.27	0.51	0.56
Eating Quality Sensory Analysis										
Appearance	0.81	-0.54	0.70	0.55	0.87	0.93	-0.75	0.61	-0.78	0.56
Tenderness	-0.09	0.55	-0.56	-0.55	-0.63	-0.93	0.35	-0.61	0.20	-0.72
Oxidation flavour	-0.41	-0.93	-0.31	0.93	-0.74	0.95	0.18	0.92	0.23	0.93
Liking of flavour	0.83	-0.94	0.74	0.94	0.88	0.96	-0.78	0.94	-0.80	0.94
Overall acceptability	0.81	0.50	0.68	-0.52	0.87	-0.93	-0.75	-0.50	-0.77	-0.71

Significance of regression coefficients; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

unaffected by level and duration of feeding of dietary L/F. Dietary supplementation where the L/F extract is subjected to porcine digestive and metabolic processes, may serve to eliminate possible negative organoleptic detection of flavourants by consumers. This indicates that seaweed extracts may be incorporated into porcine tissues via the animal's diet without damaging consumer preferences for pork meat.

5.3.7 Determination of L/F mechanism of action on lipid stability in pork muscle

The mechanism of antioxidant activity of L/F in pork meat may be attributed to one or a number of chemical reactions or pathways such as immunomodulatory activity in the gut, influence on the fatty acid composition as well as antioxidant activity (radical scavenging and ferrous ion chelating) in the muscle.

5.3.7.1 Improvement of porcine gut health

When used as feed additives in pig diets, components of the L/F extract have the potential to enhance porcine gut health by improving the immune system and altering the intestinal microbiota before being absorbed and deposited in the muscle (Gahan et al., 2009). The immune function is specially linked to the release of reactive oxygen species (ROS), the excess of which must be eliminated by endogenous antioxidant defences. ROS which are not counteracted by the antioxidant defences of the cell can become a source of damage to DNA, lipids and proteins in the animal. Molecular damage resulting from oxidative stress can lead to significant damage to cell structures and even moderate oxidation can trigger cell death (De la Fuente, 2002). Immunomodulatory activities of L/F, have been reported, in mammals through modification of macrophage activity resulting in increased immune function (Castro et al., 2004; Li, et al., 2008). It is postulated that the lipid antioxidant activity of L/F in pork meat may be mediated through enhanced immune function as a result of dietary polysaccharide supplementation.

5.3.7.2 Changes in fatty acid composition of pork muscle

Lynch et al. (2010) reported that dietary supplementation of L/F influenced short chain fatty acid (SCFA) production in pigs. Polysaccharides such as L/F undergo fermentation in the cecum and large intestine by the anaerobic cecal and colonic microbiota resulting in multiple groups of metabolites of which, SCFA are the major group. The most abundant SCFA are acetate, propionate and butyrate (den Besten et al., 2013b). SCFA can be transported from the intestinal lumen into the blood and taken up by organs where they act as substrates or signal molecules (Wong et al., 2006). SCFA, in particular acetate, have been linked to the synthesis of cholesterol and long chain fatty acids as well as being a substrate for glutamine and glutamate synthesis (den Besten et al., 2013a). Therefore the potential exists for dietary L/F to influence the FA profile of pork meat.

The fatty acid composition of pork meat for all dietary treatments is presented in Table 5.8. Susceptibility to lipid oxidation in pork meat is largely determined by the level of unsaturated fatty acids and pro-oxidants present in the muscle (Decker, et al., 2000). The fatty acid composition of the control is comparable to previously reported values for fresh pork meat (Wood et al., 2004). L/F_{900-3} was lower ($p < 0.05$) in stearic acid; L/F_{900-6} was lower ($p < 0.05$) in stearic and arachidic acid relative to the control, resulting in a decreased ($p < 0.05$) total level of saturated ($\sum$SFA) fatty acids in L/F_{900-6}. Trends indicated that all pigs fed dietary L/F had lower levels of saturated ($\sum$SFA) fatty acids, however results were not statistically significant. In pork meat, stearic acid content has been linked to fat hardness due to the saturated nature of this fatty acid. However, in the present study, the tenderness sensory descriptor of cooked LTL muscle was unaffected by stearic acid level. Trends also suggested that higher levels of polyunsaturated ($\sum$PUFA)

Table 5.8. Effect of dietary laminarin/fucoidan (L/F) on fatty acid composition in fresh *longissimus thoracis et lumborum* (LTL).

	Treatment				
	Control	**L/F$_{450-3}$**	**L/F$_{450-6}$**	**L/F$_{900-3}$**	**L/F$_{900-6}$**
Lauric C12:0	0.12 ± 0.08^{a}	0.12 ± 0.05^{a}	0.13 ± 0.05^{a}	0.11 ± 0.03^{a}	0.12 ± 0.06^{a}
Myristic C14:0	0.83 ± 0.20^{a}	0.84 ± 0.30^{a}	0.85 ± 0.18^{a}	0.87 ± 0.13^{a}	0.89 ± 0.13^{a}
Palmitic C16:0	22.18 ± 0.73^{a}	21.95 ± 1.51^{a}	22.09 ± 0.63^{a}	21.91 ± 0.47^{a}	21.56 ± 0.97^{a}
Stearic C18:0	13.84 ± 1.29^{a}	12.97 ± 0.76^{ac}	13.21 ± 0.38^{ad}	12.59 ± 0.40^{bcd}	11.65 ± 0.45^{b}
Arachidic C20:0	0.26 ± 0.05^{a}	0.22 ± 0.04^{ab}	0.23 ± 0.03^{ab}	0.22 ± 0.01^{ab}	0.17 ± 0.09^{b}
ΣSFA	37.24 ± 2.08^{a}	36.10 ± 1.65^{ab}	36.51 ± 1.02^{ab}	35.69 ± 0.73^{ab}	34.39 ± 1.25^{b}
t-Palmitoleic C16:1 *trans*	0.28 ± 0.02^{a}	0.28 ± 0.06^{a}	0.29 ± 0.04^{a}	0.30 ± 0.03^{a}	0.27 ± 0.04^{a}
Palmitoleic C16:1 *cis*	1.71 ± 0.43^{a}	1.80 ± 0.73^{a}	1.83 ± 0.27^{a}	2.04 ± 0.44^{a}	2.25 ± 0.42^{a}
Elaidic C18:1 *trans*	0.37 ± 0.10^{a}	0.37 ± 0.18^{a}	0.38 ± 0.13^{a}	0.38 ± 0.10^{a}	0.30 ± 0.09^{a}
Oleic C18:1 (*n*-9)	31.11 ± 5.35^{a}	27.35 ± 6.63^{a}	28.92 ± 3.54^{a}	29.36 ± 3.76^{a}	32.40 ± 4.86^{a}
Vaccenic C18:1 (*n*-7)	2.98 ± 0.66^{a}	3.00 ± 0.55^{a}	3.50 ± 0.17^{a}	3.29 ± 0.25^{a}	3.58 ± 0.22^{a}
ΣMUFA	36.46 ± 5.76^{a}	32.77 ± 7.48^{a}	34.92 ± 3.68^{a}	35.37 ± 3.90^{a}	38.81 ± 5.19^{a}
Linoleic C18:2 (*n*-6)	14.85 ± 3.85^{a}	17.73 ± 4.00^{a}	15.67 ± 2.14^{a}	16.43 ± 2.41^{a}	16.28 ± 3.61^{a}
γ-Linolenic C18:3 (*n*-6)	0.05 ± 0.04^{a}	0.11 ± 0.04^{a}	0.09 ± 0.05^{a}	0.07 ± 0.04^{a}	0.06 ± 0.03^{a}
α-Linolenic C18:3 (*n*-3)	0.77 ± 0.16^{a}	0.86 ± 0.11^{a}	0.83 ± 0.19^{a}	0.92 ± 0.20^{a}	0.86 ± 0.15^{a}
Dihomo-γ-linolenic C20:3 (*n*-6)	0.25 ± 0.06^{a}	0.35 ± 0.13^{a}	0.32 ± 0.07^{a}	0.28 ± 0.07^{a}	0.28 ± 0.07^{a}
Arachidonic C20:4 (*n*-6)	2.56 ± 1.11^{a}	3.39 ± 1.56^{a}	3.16 ± 1.02^{a}	2.85 ± 0.88^{a}	2.67 ± 1.02^{a}
Eicosapentaenoic C20:5 (*n*-3)	0.17 ± 0.13^{a}	0.19 ± 0.10^{a}	0.18 ± 0.06^{a}	0.17 ± 0.06^{a}	0.13 ± 0.09^{a}
Docosatetraenoic C22:4 (*n*-6)	0.34 ± 0.15^{a}	0.46 ± 0.23^{a}	0.41 ± 0.10^{a}	0.40 ± 0.16^{a}	0.35 ± 0.12^{a}
Docosapentaenoic C22:5 (*n*-3)	0.39 ± 0.17^{a}	0.57 ± 0.26^{a}	0.54 ± 0.17^{a}	0.47 ± 0.15^{a}	0.45 ± 0.17^{a}
Docosahexaenoic C22:6 (*n*-3)	0.07 ± 0.03^{a}	0.12 ± 0.07^{a}	0.07 ± 0.09^{a}	0.08 ± 0.05^{a}	0.08 ± 0.06^{a}
ΣPUFA	19.44 ± 5.29^{a}	23.77 ± 6.19^{a}	21.28 ± 3.42^{a}	21.67 ± 3.31^{a}	21.17 ± 4.93^{a}
P:S ratio$^{\#}$	0.43 ± 0.13^{a}	0.52 ± 0.13^{a}	0.46 ± 0.07^{a}	0.49 ± 0.07^{a}	0.50 ± 0.12^{a}
18:2*n*-6:18:3*n*-3	19.89 ± 6.49^{a}	21.36 ± 7.26^{a}	20.12 ± 7.57^{a}	18.56 ± 4.53^{a}	19.24 ± 4.47^{a}
Σ*n*-6:Σ*n*-3	13.48 ± 1.31^{a}	13.49 ± 1.63^{a}	12.64 ± 1.68^{a}	12.86 ± 1.43^{a}	13.52 ± 1.69^{a}

$^{\#}$P:S ratio = (C18:2*n*-6 + C18:3*n*-3) / (C12:0 + C14:0 + C16:0 + C18:0).

^{ab}Within each fatty acid, mean values (± standard deviation) in the same row bearing different superscripts are significantly different, $p < 0.05$.

fatty acids and higher polyunsaturated:saturated (P:S) ratios in meat from all dietary seaweed treatments relative to the control, however results were not statistically significant. L/F_{900-6} displayed enhanced lipid stability in addition to significantly lower ($p < 0.05$) saturated fatty acids suggesting dietary addition of L/F beneficially influenced the fatty acid profile of LTL muscle without detrimental effects on lipid stability. This shift in the ratio of P:S may benefit consumers seeking meat with reduced saturated fat levels.

5.3.7.3 Free radical scavenging activity of L/F extract in pork muscle

The DPPH free radical scavenging activity of laminarin, fucoidan and a variety of brown seaweeds (i.e. *Fucus vesiculosus*, *Laminaria japonica*, *Sargassum plagiophyllum*, *Sargassum vulgare*) has been reported previously (Choi, et al., 2011; Dore et al., 2013; Suresh et al., 2012; Vo & Kim, 2013). Free radical scavenging activity and protection against ROS is a potential mechanism of action of dietary L/F deposited in the muscle tissue. The free radical scavenging activity of fresh pork from the dietary treatments (L/F_{450-3}; L/F_{450-6}; L/F_{900-3}; L/F_{900-6}) ranged from 38.92 to 43.45% and was similar to the control (39.49%) (Figure 5.1). A spray-dried seaweed extract containing L/F and Trolox were directly added to pork meat (positive controls) to determine *in vitro* radical scavenging activity. Direct addition of L/F (1000 and 3000 µg/ml) and Trolox (1000 µg/ml) to pork meat resulted 57.2%, 63.3% and 83.2% free radical scavenging activity, respectively. The antioxidant activity of fucoidan has been related to the sulphate content, position, molecular weight and sugar composition (Sinurat & Marraskuranto, 2013). In the present study, the seaweed extract exhibited free-radical scavenging activity *in vitro*, however, scavenging activity was not detected in pork meat following

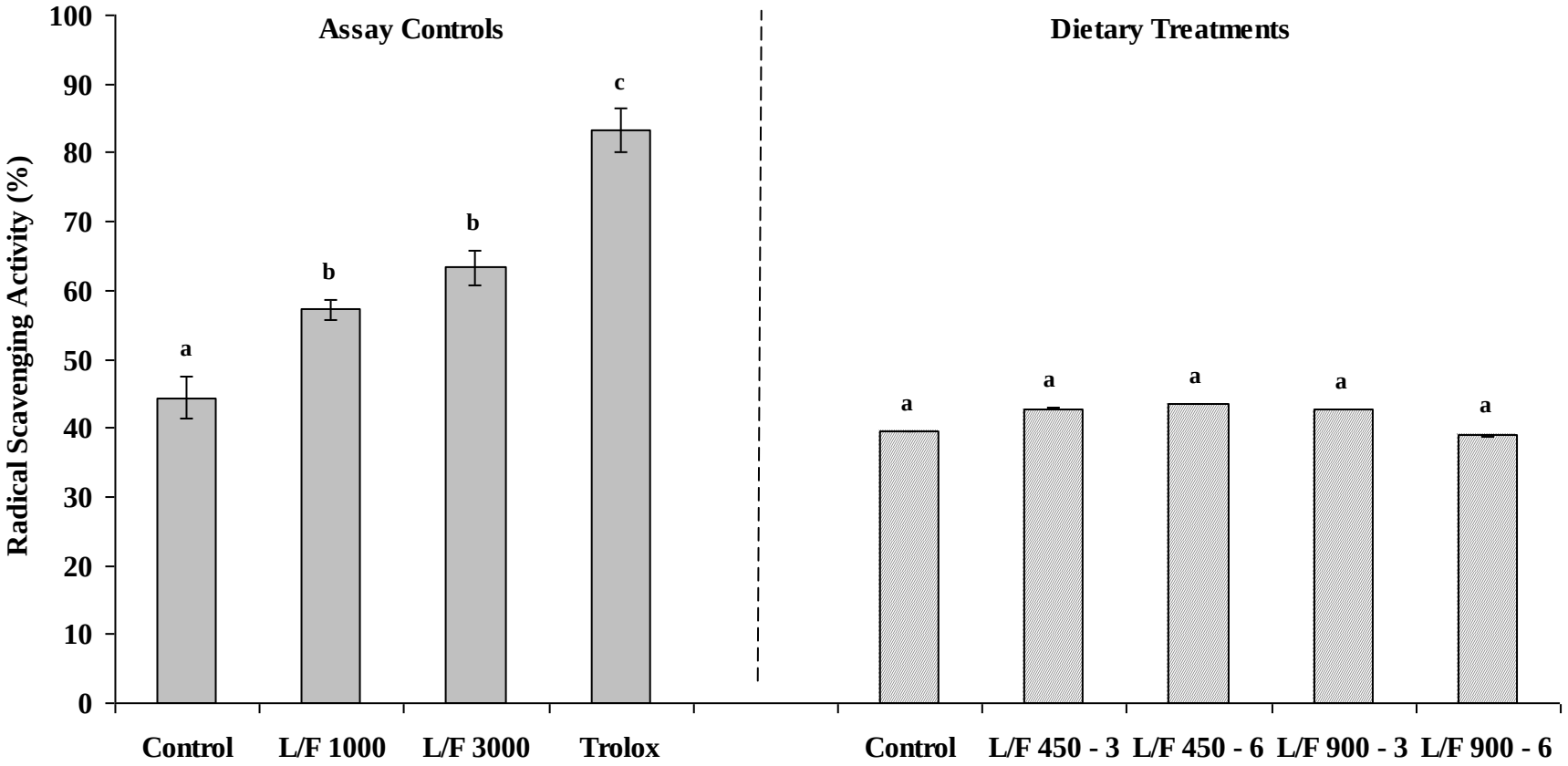

Figure 5.1. Effect of dietary laminarin/fucoidan (L/F) on DPPH free radical scavenging in fresh *longissimus thoracis et lumborum* (LTL) muscle. [abc]Within each dataset (assay controls/dietary treatment), mean values (± standard deviation error bars) bearing different superscripts are significantly different, $p < 0.05$. (▢), Direct addition to meat homogenates; (▨), Dietary treatments.

dietary supplementation with L/F. The lack of free-radical scavenging activity is potentially due to biotransformation of L/F during ingestion and absorption by the porcine gut before deposition in the muscle resulting in compounds un-reactive with the DPPH radical.

5.3.7.4 Ferrous ion chelating activity of L/F extract in pork muscle

Transition metals present in muscle foods such as iron are known to initiate and accelerate lipid oxidation in food systems. Low to moderate ferrous ion chelating activities of brown seaweeds (*Sargassum filipendula* and *Laminaria japonica*) have been previously reported, and activity was attributed to the presence of sulphated polysaccharides (Costa et al., 2011; Wang et al., 2008). Analysis of the L/F extract (rich in sulphated polysaccharides) (1000 and 3000 μg/ml) utilised in the present study, indicated no FICA whereas EDTA (positive control) resulted in 90.95% FICA. Iron chelating activity is dependent on the complex structural characteristics of seaweed polysaccharides (Mak et al., 2013). The L/F extract may undergo structural modification (biotransformation) during porcine digestion and potentially exhibit antioxidant activity mediated through ferrous ion chelating activity in pork meat. FICA of pork meat fractions from each dietary treatment ranged from 17.65 -25.10% (similar to the control) (Figure 5.2). Results indicated that dietary L/F did not influence FICA in LTL muscle and antioxidant activity of L/F is most likely due to a combination of the proposed mechanisms discussed.

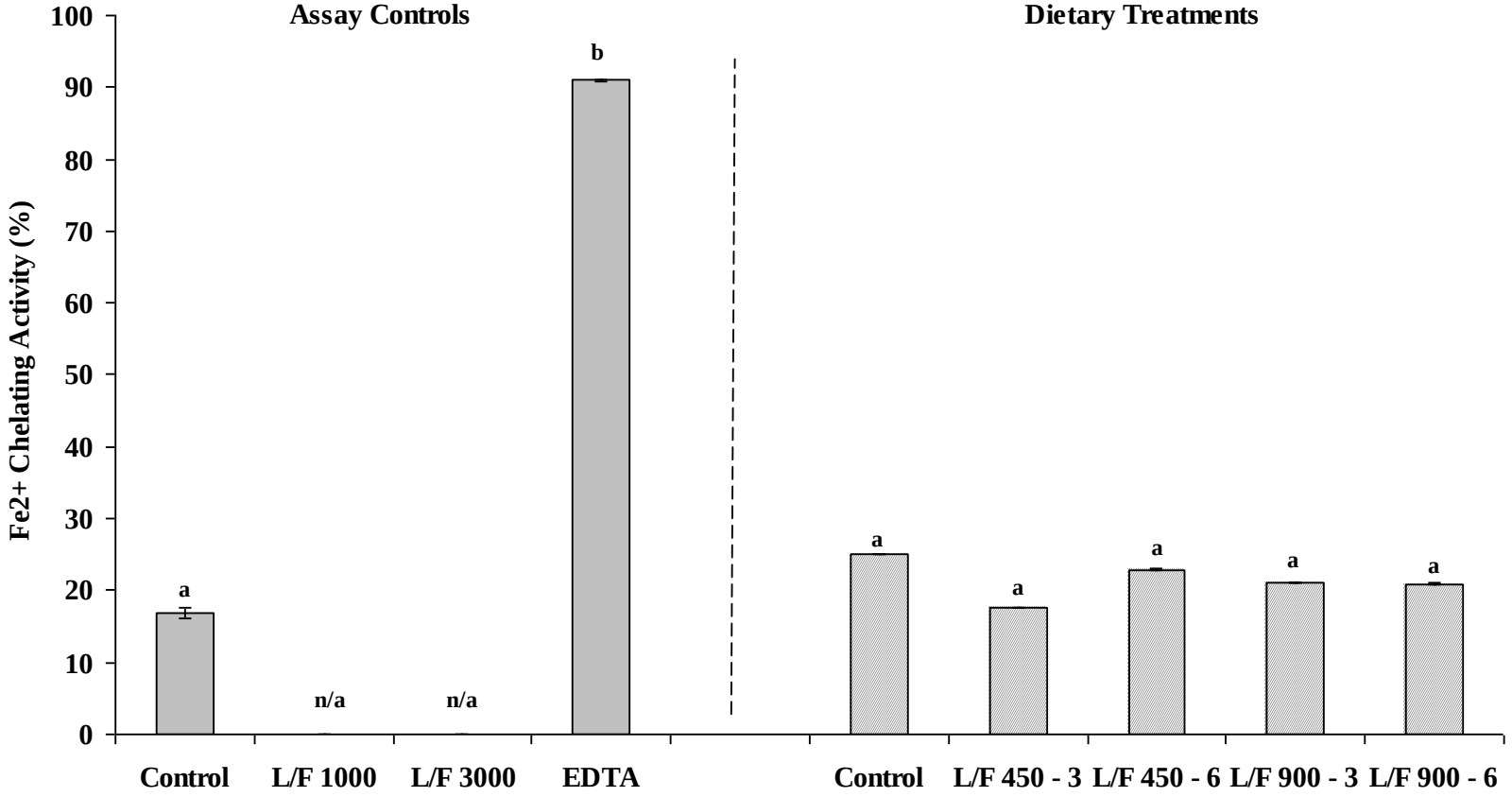

Figure 5.2. Effect of dietary laminarin/fucoidan (L/F) on ferrous ion chelating activity (FICA) in fresh *longissimus thoracis et lumborum* (LTL) muscle. [ab]Within each dataset (assay controls/dietary treatment), mean values (± standard deviation error bars) bearing different superscripts are significantly different, $p < 0.05$. ( ), Direct addition to meat homogenates; ( ), Dietary treatments. [n/a] No activity.

5.4 CONCLUSIONS

Dietary supplementation of L/F at 3 weeks, irrespective of level, resulted in lower levels of lipid oxidation due to deposition of marine-derived bioactive antioxidant components in LTL muscle. Antioxidant response factor, dependant on duration of supplementation, was most likely attributed to a number of mechanisms. Dietary L/F reduced total level of saturated (∑SFA) fatty acids in L/F_{900-6} and significantly lowered lipid oxidation in LTL muscle (on day 11 and 14 of storage) in L/F_{450-3} and L/F_{900-3}. Due to complexity of the extract and porcine metabolic pathways, it is unclear if free radical scavenging abilities of the extract were responsible for the antioxidant activity observed in LTL muscle. Meat quality enhancing effects of seaweed polysaccharides may be mediated through health promoting effects of gut-associated immunity. The improved fatty acid profile with enhanced lipid stability of pork meat without impact on tenderness, flavour or other sensory properties suggests dietary supplementation of seaweed extracts containing laminarin and fucoidan could result in an enhanced pork meat product.

CHAPTER 6

Effect of a brown seaweed (*Laminaria digitata*) extract containing laminarin and fucoidan on the quality and shelf-life of fresh and cooked minced pork patties

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ABSTRACT

A spray-dried seaweed extract containing laminarin (L, 9.3%) and fucoidan (F, 7.8%) (L/F extract) from brown seaweed (*Laminaria digitata*) was added directly to minced pork (*longissimus thoracis et lumborum*) (LTL) at levels of 0.01%, 0.1% and 0.5% (w/w). Fresh and cooked minced pork patties were stored in modified atmosphere packs containing 80% O_2 : 20% CO_2 and 70% N_2 : 30% CO_2, respectively, for up to 14 days at 4ºC. The L/F extract (0.5%) exerted a lipid pro-oxidant activity in fresh patties on each measurement day. The L/F extract (0.5%) significantly decreased ($p < 0.05$) lipid oxidation in cooked patties on days 1, 4 and 14 of storage. The L/F extract had no effect on the microbiological status, pH, water holding capacity (WHC) or cook loss of patties. Sensory panellists were unable to distinguish between pork patties containing 0.01% L/F and the control. Further research will focus on the use of refined purified seaweed extracts in functional meat products.

6.1 INTRODUCTION

The use of seaweed (macroalgae) or seaweed extracts as food additives is growing in popularity due to the vast range of functional properties they impart in food products. Seaweeds contain high proportions of polysaccharides, proteins, minerals, vitamins and a low lipid content (Gómez-Ordóñez et al., 2010). Seaweed polysaccharides are a potential source of soluble and insoluble dietary fibre. Soluble seaweed polysaccharides exhibit higher water holding capacity than cellulosic (insoluble) fibres. Soluble dietary fibres demonstrate the ability to increase viscosity, form gels and/or act as emulsifiers and are also characterised by a capacity to reduce both glycemic response and plasma cholesterol in humans (Elleuch et al., 2011; Venugopal, 2008).

The cell walls of brown seaweed (*Phaeophyta*) contain polysaccharide compounds such as laminarin and fucoidan (soluble fibres). Laminarin, a β-polymer of glucose, is the main storage polysaccharide in algae. Fucoidan, a sulphated heteropolysaccharide, is composed primarily of L-fucose and protects seaweed from desiccation (Bocanegra et al., 2009, Anastasakis et al., 2011). Seaweed polysaccharides, including laminarin and fucoidan are reported to possess antioxidant (Choi et al., 2011), anti-tumour, anticoagulant, antiviral, and antibacterial activities (Costa et al., 2010; Wang et al., 2007).

The development of functional meats with enhanced physiochemical and health-promoting properties may be achieved by adding seaweeds or seaweed extracts containing bioactive components into meat and meat products. Bioactive compounds may be incorporated by supplementation of animal diets or by direct addition during meat processing (Khan et al., 2011). In Chapters 4 and 5, supplementation of pig diets with brown seaweed (*Laminaria digitata*) extracts containing laminarin and fucoidan enhanced the oxidative stability of fresh pork meat. Similarly, Diaz-Rubio et al. (2011) reported

that the direct addition of dietary fibre from *Fucus vesiculosus* inhibited lipid oxidation in fish mince muscle.

Recent scientific studies have also examined the functionality of macroalgae (powdered/dried *Himanthalia elongata*, *Undaria pinnatifida*, *Porphyra umbilicalis*, and *Laminaria japonica*) in processed meat products, such as beef or pork burgers/patties, frankfurters, restructured poultry steaks, and pork meat emulsion systems (Choi et al., 2012; Cofrades et al., 2011; López-López et al., 2009c; López-López et al., 2010). Pigments present in seaweed can influence meat product colour depending on the seaweed/extract type and concentration added (Choi et al., 2012; Jiménez-Colmenero et al., 2010; López-López et al., 2009c). Acidic seaweed components such as fucoidan and alginic acid decreased the pH of meat products containing powdered seaweeds (Choi et al., 2012; Cofrades et al., 2008). The antioxidant capacity (FRAP) of pork meat emulsion systems increased due to the high phenolic content of powdered seaweed added (López-López et al., 2009a). Algal oils have been used to enhance the lipid profile of frankfurters (López-López et al., 2009c). Soluble and insoluble dietary fibre from macroalgae increased cooking yields, improve texture, fat/water binding and emulsion stability and decreased costs in meat product formulations (Fernández-Martín et al., 2009, Jiménez-Colmenero et al., 2010). Seaweeds contain a high concentration of minerals and therefore may also have potential for use as salt replacers in processed meat products (Cofrades et al., 2011).

Studies on the addition of seaweed polysaccharides to meat products are limited and merit investigation. The objective of this study was to examine the effect of the direct addition of a spray-dried seaweed (*Laminaria digitata*) extract containing laminarin and fucoidan (L/F) on the quality and shelf-life of minced pork (*longissimus thoracis et lumborum* (LTL)) stored in modified atmosphere packs. Quality parameters examined

include colour, lipid oxidation (fresh and cooked), microbiology, pH, water holding capacity (WHC), texture profile analysis (TPA), cook loss, and sensory properties during refrigerated storage at 4ºC.

6.2 MATERIALS AND METHODS

6.2.1 Reagents

All chemicals used were 'AnalaR' grade obtained from Sigma-Aldrich Ireland Ltd., Arklow, Co. Wicklow, Ireland, Oxoid Ltd., Basingstoke, Hampshire, England and Merck KGaA, Darmstadt, Germany. Fresh pork muscle (*longissimus thoracis et lumborum*) (LTL) was supplied by Ballyburden Meat Processors, Ballincollig, Co. Cork, Ireland. A spray-dried seaweed extract, containing laminarin (L) and fucoidan (F) (L/F extract) was manufactured by Bioatlantis, Tralee, Co. Kerry, Ireland. The L/F extract was prepared from brown seaweed (*Laminaria digitata*), using an acid extraction technique, the details of which are confidential. The composition of the L/F extract was 94.03% total solids, 68.6% ash, 0.64% protein, 9.3% laminarin, 7.8% fucoidan, and 8.3% mannitol. The ash component of the L/F extract was composed of 15 g/kg DM Ca, 10 g/kg DM Na, 10 g/kg DM K, 10 g/kg DM S, 250 mg/kg DM iodine, 250 mg/kg DM Fe, 20 mg/kg DM Cu and 50 mg/kg DM Zn. Tea catechins (TC) (81.43%) extracted from green tea was supplied by New Kinglong Natural Products Co. Ltd, Hunan, China.

6.2.2 Salt determination

The salt content of the L/F extract was measured using the mercuric nitrate titration method of Dubsky and Trtilek as described by Roberts (1936). The L/F extract (5 g) was dissolved in 40 ml of distilled water. Samples were filtered through Whatman No. 1 filter paper with glass wool into a 100 ml volumetric flask and made up to volume with distilled water. Filtrate (25 ml) plus 15 ml of 0.01N HNO_3 and 1 ml of diphenylcarbazone indicator was titrated against 0.023N $Hg(NO_3)_2$ standardised with 25 ml of 0.25% NaCl. A purple colour denoted the titration end-point. The salt percentage was calculated using the following equation:

$$\%Salt = ml\ Hg(NO_3)_2 / 5$$

The salt content of the extract was 1% ± 0.1%. In the minced pork studies, a salt control (ST) was used to determine the effect of the salt contained in the L/F extract on the quality parameters examined. The addition of salt (50 mg/kg) to fresh minced pork (ST) was equivalent to the salt content of 0.5% L/F pork treatment.

6.2.3 Pork processing and packaging

Longissimus thoracis et lumborum (LTL) muscles were trimmed of visible fat and connective tissue and minced twice through a plate with 4 mm holes (Model P114L, Talsa, Valencia, Spain). Following mincing, fresh pork was assigned to one of six treatments: untreated pork (Control); 0.005% salt (salt control) (ST); 0.1% tea catechin (positive lipid oxidation control) (TC); pork plus increasing amounts of the L/F extract: 0.01% L/F, 0.1% L/F and 0.5% L/F. ST, TC and L/F were dissolved in water, immediately added to raw minced pork (5% v/w) and mixed vigorously.

Minced pork from each treatment was formed into patties (100 g portions) using a meat former (Ministeak burger maker, O.L Smith Co. Ltd., Italy), placed in low oxygen permeable (<1cm^3/m^2/24 hr at STP) polystyrene/ethylvinylalcohol/polyethylene trays and covered using a low oxygen permeable (3 cm^3/m^2/24 hr at STP) laminated barrier film with a polyolefin heat-sealable layer. Using modified atmosphere packaging (MAP) technology, the trays were flushed with 80% O_2 : 20% CO_2 using a vacuum-sealing unit (VS 100, Gustav Müller and Co. KG, Bad Homburg, Germany) equipped with a gas mixer (Witt-Gasetechnik GmbH and Co. KG, Witten, Germany) and heat-sealed.

In the cooked pork study, minced pork patties (0.5% L/F) were placed on aluminium foil lined trays and cooked at 180°C for 20 min in a fan-assisted convection oven (Zanussi Professional, Model 10 GN1/1, Conegliano, Italy) until an internal

temperature of 72°C was reached. Cooked patties were placed in MAP trays and flushed with 70% N_2 : 30% CO_2 and packaged as described previously. Fresh and cooked pork patties were stored for up to 14 days under fluorescent lighting conditions (approximately 660 lx) at 4°C. The gas atmosphere (% O_2 and % CO_2) in the MAP was checked using a CheckMate 9900 (PBI-DanSensor, Denmark). The % N_2 was calculated by difference. Immediately after gas flushing, fresh pork MAP trays contained 77.15 ± 0.37% O_2 and 22.73 ± 0.33% CO_2 and the gas composition after 14 days was 67.75 ± 3.82% O_2 and 30.00 ± 3.87% CO_2. In cooked pork MAP trays the gas composition was 67.46 ± 0.82% N_2 and 32.13 ± 0.49% CO_2 directly after gas flushing and 68.79 ± 0.14% N_2 and 31.10% ± 0.08% CO_2 after 14 days of storage.

6.2.4 Measurement of pork quality and shelf-life parameters

Pork pH, colour (CIE 'L*' lightness, 'a*' redness and 'b*' yellowness values), lipid oxidation (2-thiobarbituric acid reactive substances (TBARS)), and microbiological analysis (mesophilic and psychrotrophic total viable counts) were measured at intervals during storage as described in Chapter 4 (sections 4.2.5-4.2.8). The pH, colour measurements and lipid oxidation of fresh minced pork patties were recorded on days 1, 4, 7, 11 and 14 of storage. In cooked minced pork patties stored in MAP, lipid oxidation was measured on days 1, 4, 7, 11 and 14 of storage. Microbiological analysis of fresh minced pork patties was carried out on days 1, 5, 8 and 12 of storage.

6.2.5 Water holding capacity

Water holding capacity (WHC) was measured as described by Lianji & Chen (1989). Approximately 10 g of fresh minced pork was weighed into glass jars, covered with aluminium foil and heated in a water bath for 10 min at 90°C. After heating,

samples were carefully removed from each jar using forceps, wrapped in cheesecloth, and placed in 30 ml centrifuge tubes lined with cotton wool at the base of each tube. The samples were centrifuged (Beckman J2-21, Beckman Instruments Inc., CA, USA) at 13,440 g for 10 min at 4°C. Following centrifugation, the cheesecloth was removed and samples were reweighed. Measurements of the moisture content (M) of pork samples were carried out on the Smart Trac5 rapid moisture/fat analyser (CEM Corporation). The percentage WHC was calculated using the following equation:

$$\% \text{ WHC} = [1 - [(B - A) / (B \times M)]] \times 100$$

Where B denotes the weight of sample before heating; A, the weight of sample after heating and centrifuging; and M the % moisture of the sample (CEM). WHC was measured on days 2 and 7 of storage.

6.2.6 Texture profile analysis

Texture profile analysis (TPA) was carried out on cooked pork patties based on a method described by Bourne (1978). Cooked patties (20mm x 20mm x 20mm) were axially compressed to 50% of their original height in a two-cycle compression test with an aluminium cylinder probe of 2 cm diameter using a texture analyser (TA.XT2i Texture Analyser, Stable Micro Systems, UK). Force time deformation curves were obtained using a 5 kN load cell applied at a cross speed of 50 mm/min. Attributes were calculated as follows: hardness (N), peak force required for first compression; springiness (mm), distance sample recovers after first compression; adhesiveness (N), the negative force area for the first bite representing the work necessary to pull the compressing plunger away from the sample; cohesiveness (dimensionless), ratio of positive force area during the second compression; gumminess (N), the product of hardness and cohesiveness;

chewiness (N x mm), the product of gumminess and springiness. TPA was carried out on days 2 and 7 comparable with sensory analysis measurement days.

6.2.7 Cook loss

The weight of minced pork patties was recorded before and after cooking. Patties were cooled for 1 hr before re-weighing. Cook loss was measured on days 2 and 7 of storage and calculated using the following equation:

$$\% \text{ cook loss} = [(\text{raw weight} - \text{cooked weight}) / \text{raw weight}] \times 100$$

6.2.8 Sensory evaluation

Sensory analysis ('visual' and 'eating quality') of fresh pork patties stored in 80% O_2 : 20% CO_2 was performed by 52 naïve assessors on days 2 and 7 of storage following the method of O'Sullivan et al. (2003). On day 8, the bacterial count of the fresh pork patties (> 6 $\log_{10}$ cfu/g) was considered too high for further sensory evaluation. 'Visual' sensory analysis descriptors were redness, brownness, drip, package quality, purchasing appeal, and overall acceptability. 'Eating quality' sensory analysis descriptors were appearance, tenderness, oxidation flavour, liking of flavour 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).

Pork patties were cooked for sensory analysis in a Zanussi oven at 180ºC for 20 min until an internal meat temperature of 72ºC was reached. Following cooking, patties were cooled and cut into 2 cm x 2 cm cubes and identified with random three-digit codes. Sample presentation order was randomised to prevent any flavour carryover effects (MacFie et al., 1989). Prior to serving to panellists, pork samples were re-heated in a microwave for 10 sec to release the meat odour and flavour. Sensory analysis was

undertaken in the panel booths at the University sensory laboratory in accordance with the ISO (2007) International Standard guidelines. Assessors were also provided with water and crackers to cleanse their palate between samples. Results for sensory analysis scores were measured in centimetres (cm) and scores were statistically analysed using ANOVA-partial least squares regression (APLSR). Results were presented as significance of regression coefficients, analysed by jack-knife testing.

6.2.9 Statistical analysis

All analyses were performed in duplicate and three independent experimental trials were carried out. A full repeated measures ANOVA was conducted to investigate the effects of L/F concentration, time and their interactions. L/F concentration represented the 'between-subjects' factor and the effect of time was measured using the 'within-subjects' factor. Tukey's test was used to adjust for multiple comparisons between treatment means. The analysis was carried out using SPSS 18.0 for Windows (SPSS, Chicago, IL, USA) software package.

'Visual' and 'eating quality' sensory data was analysed with ANOVA-Partial Least Squares Regression (APLSR) to process the mean data accumulated from the 52 test subjects. 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. In these models assessor and session level effects were removed using level correction. The validated model explained variance was -0.51% on day 2 and 6.27% on day 7 and the calibrated variance was 2.38% on day 2 and 8.97% on day 7. 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 the Unscrambler Software, version 9.8 (CAMO ASA, Trondheim, Norway).

6.3 RESULTS AND DISCUSSION

6.3.1 Colour stability of fresh minced pork

Trends indicated that the '*L**' lightness values increased in fresh pork patties (0.01% L/F, 0.1% L/F, and 0.5% L/F) over the 14 day storage period and patties were unaffected by L/F extract concentration, however results were only statistically significant ($p < 0.05$) for 0.5% L/F (Table 6.1). In a similar study, the addition of powdered *L. japonica* (1%, 3,% and 5%) decreased the lightness values of uncooked reduced fat pork patties due to the brown and yellow pigments such as chlorophylls, phycophine, and xanthophylls present in the extract (Choi et al., 2012).

The '*a**' redness values of pork patties significantly ($p < 0.05$) decreased as a function of storage time. The L/F extract (0.1% L/F, and 0.5% L/F) reduced the surface redness ('a*' values) of pork patties on days 7, 11 and 14 of storage as a function of L/F concentration, however results were not statistically significant ($p > 0.05$) (Table 6.1). Similarly Cofrades et al. (2008) reported that the addition of dried *Himanthalia elongata*, *Undaria pinnatifida* and *Porphyra umbilicalis* (2.5% and 5%) reduced the '*a**' redness values of a pork meat gel/emulsion in a concentration dependent manner.

Previous studies have linked oxymyoglobin oxidation and discoloration in meat to lipid oxidation, with an increase in one resulting in a similar increase in the other due to a number of proposed mechanisms (O'Grady et al., 2001). At the highest level of L/F addition (0.5%), levels of lipid oxidation in pork patties were higher than the control on days 4, 7, 11 and 14 of storage (Table 6.2). Increased levels of lipid oxidation may subsequently promote oxymyoglobin oxidation which may explain the observed decreased surface redness values of pork patties in the present study.

In addition, decreased surface redness may be attributed to interactions between pork meat constituents and the added seaweed extract. Previous studies have indicated

Table 6.1. Effect of L/F extract addition on surface lightness ('*L**' value), redness ('*a**' value) and yellowness ('*b**' value) values of fresh pork patties stored in modified atmosphere packs (80% O_2 : 20% CO_2) for up to 14 days at 4°C.

Treatment		Storage time at 4°C, days				
	Parameter	**1**	**4**	**7**	**11**	**14**
Control	Lightness	56.77 ± 2.25^{aA}	58.30 ± 3.12^{aA}	58.86 ± 1.58^{aA}	60.12 ± 1.49^{aA}	60.36 ± 2.19^{aA}
0.01% L/F	*L**	56.76 ± 2.19^{aA}	58.20 ± 3.05^{aA}	60.20 ± 0.99^{aA}	61.10 ± 0.89^{aA}	60.81 ± 2.17^{aA}
0.1% L/F		56.66 ± 3.32^{aA}	57.10 ± 3.83^{aA}	59.15 ± 1.75^{aA}	61.09 ± 1.11^{aA}	60.88 ± 1.44^{aA}
0.5% L/F		55.28 ± 2.31^{aA}	56.24 ± 3.11^{aA}	59.94 ± 1.28^{aAB}	62.19 ± 0.58^{aB}	62.35 ± 1.45^{aB}
ST*		56.19 ± 1.80^{aA}	56.15 ± 1.51^{aA}	57.88 ± 1.09^{aAB}	60.73 ± 2.50^{aAB}	61.94 ± 2.69^{aB}
Control	Redness	8.78 ± 0.48^{aA}	6.87 ± 0.53^{aAB}	4.89 ± 2.00^{aBC}	3.29 ± 1.76^{aBC}	2.40 ± 1.34^{aC}
0.01% L/F	*a**	8.68 ± 0.53^{aA}	6.74 ± 0.51^{aAB}	4.72 ± 1.99^{aBC}	3.26 ± 1.86^{aBC}	2.14 ± 1.05^{aC}
0.1% L/F		8.54 ± 0.54^{aA}	6.86 ± 0.75^{aA}	3.80 ± 1.92^{aB}	1.56 ± 0.78^{aBC}	0.94 ± 0.67^{aC}
0.5% L/F		7.79 ± 0.52^{aA}	6.15 ± 0.77^{aA}	2.47 ± 1.89^{aB}	0.41 ± 0.72^{aB}	-0.03 ± 0.72^{aB}
ST		8.92 ± 0.31^{aA}	7.33 ± 1.03^{aAB}	5.21 ± 2.65^{aBC}	1.85 ± 0.91^{aCD}	0.33 ± 0.68^{aD}
Control	Yellowness	9.83 ± 0.34^{aA}	9.24 ± 0.20^{aA}	9.00 ± 0.16^{aA}	8.97 ± 0.48^{aA}	8.86 ± 1.02^{aA}
0.01% L/F	*b**	9.71 ± 0.33^{aA}	9.24 ± 0.29^{aA}	9.26 ± 0.34^{aA}	9.22 ± 0.41^{aA}	8.85 ± 0.66^{aA}
0.1% L/F		9.82 ± 0.07^{aA}	9.37 ± 0.29^{aA}	9.08 ± 0.25^{aA}	9.11 ± 0.67^{aA}	9.27 ± 0.53^{aA}
0.5% L/F		9.99 ± 0.27^{aA}	9.81 ± 0.03^{aA}	10.06 ± 0.51^{aA}	10.21 ± 1.02^{aA}	10.21 ± 0.40^{aA}
ST		9.85 ± 0.29^{aA}	9.36 ± 0.37^{aA}	9.13 ± 0.67^{aA}	9.76 ± 0.66^{aA}	9.99 ± 0.39^{aA}

[a]Within each parameter and storage day, mean values (± standard deviation) in the same column are not significantly different, $p > 0.05$.

[ABC]Within each treatment, mean values (± standard deviation) in the same row bearing different superscripts are significantly different, $p < 0.05$.

[*]Salt control (ST), 0.005% salt.

that polysaccharides can interact with proteins and it is generally accepted that electrostatic interactions occur between the anionic groups of a polysaccharide (seaweed polyanionic alginates) and the positively charged groups present in proteins (Imeson et al., 1977). Sulphated polysaccharides are capable of forming soluble complexes with globular proteins at pH values above the protein isoelectric point (Hill et al., 1998). Imeson (1977) determined that the presence of the anionic polysaccharides altered the spectrum from that typical of metmyoglobin to one with a decreased absorption in the Soret (~400 nm) region; in the presence of pectate and alginate the Soret absorption also occurs at lower wavelengths. Therefore the dose-dependant decrease in '*a**' redness values, of pork patties may be partially attributed to interactions between polysaccharides (L/F) present in the extract and oxymyoglobin in pork meat.

In Chapters 4 and 5, the spray-dried L/F extract, identical to that used in this chapter, did not affect the 'a*' redness values when incorporated into fresh pork via animal's diet. Therefore the manner (dietary supplementation versus direct addition) by which seaweed extracts are added to meat appears to influence the mode of action of the bioactive components present.

Trends indicated that the 'b*' yellowness values of fresh pork patties containing up to 0.1% L/F decreased over the 14 day storage period, however results were not statistically ($p > 0.05$) significant (Table 6.1). The addition of 0.5% L/F to fresh pork patties resulted in increased 'b*' yellowness values over time, however results were not statistically ($p > 0.05$) significant. Similarly, Cofrades et al. (2008) reported that dried *H. elongata* and *Undaria pinnatifida* (2.5% and 5%) increased 'b*' yellowness values in pork emulsion systems. In addition to chlorophyll, brown seaweeds contain phycophine, a brown pigment, and xanthophyll, a yellow pigment, which provides the seaweeds with a variety of shades in the yellow-dark chestnut range, including yellowish greens which can

mask the green of chlorophyll (Cofrades et al., 2008). The change in the yellowness values of the pork patties in the present study may be attributed to the addition of natural colour pigments present in the L/F extract.

6.3.2 Lipid oxidation in fresh and cooked minced pork

In general, levels of lipid oxidation in fresh pork patties followed the order: 0.5% L/F > ST > 0.1% L/F > Control > 0.01% L/F > TC (Table 6.2). No statistical difference was observed between L/F extract treatments (0.01% L/F and 0.1% L/F) and controls (control and TC) on any of the storage days. Levels of lipid oxidation were lowest in TC-containing pork patties, however results were not statistically significant ($p > 0.05$). Previous studies have demonstrated the potent antioxidant activity of TC in muscle foods (Tang et al., 2001). In pork patties containing 0.5% L/F, the extract exerted a lipid pro-oxidant activity on each measurement day ($p < 0.05$). Although results were not statistically significant ($p > 0.05$), a pro-oxidant effect was also observed in pork patties containing salt (ST) at levels equivalent to 50 mg/kg, indicating that Na present in 0.5% L/F may be responsible for catalysis of lipid oxidation in fresh pork patties. Numerous studies have demonstrated the pro-oxidant properties of salt in muscle foods (Tang et. al, 2001). In fresh meat, salt can promote the formation of hypervalent ferrylmyoglobin (or activated metmyoglobin), an initiator of lipid oxidation (Rhee & Ziprin, 2001).

The L/F extract also contained minerals such as iron (250 mg/kg DM) and copper (20 mg/kg DM) which are known to promote lipid oxidation in meat products (Bandy et al., 2001; Decker & Xu, 1998; Rhee & Ziprin, 2001). Transition metals, in particular iron, initiate lipid oxidation either directly or indirectly by facilitating the generation of other initiating factors. Metals may also play a role in the propagation of lipid oxidation by catalysing the breakdown of lipid hydroperoxides and iron is considered a major

Table 6.2. Effect of L/F extract addition on lipid oxidation (TBARS*) of fresh and cooked pork patties stored in 80% O_2 : 20% CO_2, and 70% N_2 : 30% CO_2, respectively, for up to 14 days at 4°C.

Treatment	Parameter	Storage time at 4°C, days				
		1	4	7	11	14
Control	Fresh	0.062 ± 0.02^{aA}	0.134 ± 0.02^{aA}	0.119 ± 0.05^{abcA}	0.372 ± 0.23^{abcA}	0.374 ± 0.12^{abcA}
TC†		0.043 ± 0.02^{aA}	0.117 ± 0.04^{aA}	0.038 ± 0.02^{bA}	0.097 ± 0.06^{bA}	0.129 ± 0.05^{bA}
0.01% L/F		0.059 ± 0.01^{aA}	0.139 ± 0.01^{aA}	0.129 ± 0.07^{abcA}	0.341 ± 0.23^{abcA}	0.358 ± 0.13^{abcA}
0.1% L/F		0.062 ± 0.02^{aA}	0.155 ± 0.04^{aAB}	0.207 ± 0.14^{abcAB}	0.467 ± 0.30^{abcAB}	0.582 ± 0.21^{abcB}
0.5% L/F		0.159 ± 0.04^{bA}	0.285 ± 0.08^{bA}	0.726 ± 0.47^{cA}	1.327 ± 0.72^{cA}	1.380 ± 0.77^{abcA}
ST¥		0.090 ± 0.02^{aA}	0.116 ± 0.04^{aA}	0.282 ± 0.09^{abcAB}	1.195 ± 0.64^{cAB}	1.509 ± 0.93^{cB}
Control	Cooked	1.662 ± 0.07^{aA}	1.868 ± 0.29^{aB}	1.994 ± 0.36^{aB}	2.257 ± 0.06^{aC}	2.795 ± 0.01^{aD}
0.5% L/F		1.095 ± 0.30^{bA}	1.261 ± 0.16^{bAB}	1.757 ± 0.55^{aABC}	2.187 ± 0.48^{aBC}	2.273 ± 0.13^{bC}

[ab]Within each parameter and storage day, mean values (± standard deviation) in the same column bearing different superscripts are significantly different, $p < 0.05$.
[ABCD]Within each treatment, mean values (± standard deviation) in the same row bearing different superscripts are significantly different, $p < 0.05$.
†Tea catechins (TC) (positive lipid oxidation control), 0.1%. ¥Salt control (ST), 0.005% salt. *TBARS, mg malondialdehyde (MDA)/kg pork.

catalyst of oxidative rancidity in meat (Ruiz et al., 2000). Therefore, minerals present in the L/F extract may also be responsible for the catalysis of lipid oxidation in fresh pork patties.

In Chapters 4 and 5, the supplementation of porcine diets with a spray-dried L/F extract, identical to that used in the present study, decreased lipid oxidation in fresh pork LTL muscle. It was concluded that antioxidant components of laminarin and fucoidan were deposited in LTL muscle thereby enhancing lipid stability of pork meat. Dietary supplementation of L/F extract may serve to eliminate the effects of pro-oxidant components contained in the extract presumably due to porcine digestive and metabolic processes.

In contrast to the results reported in the present study, the addition of powdered *H. elongata*, *U. pinnatifida* and *P. umbilicalis* (2.5% and 5%) increased antioxidant activity in low-salt pork meat emulsion model systems. Antioxidant activity, determined using *in vitro* antioxidant assays (FRAP and TEAC), was attributed to polyphenolic compounds present in the seaweeds (López-López et al., 2009a).

In cooked pork patties, lipid oxidation significantly ($p < 0.05$) increased as a function of storage time. Pork patties containing L/F extract (0.5%) had significantly lower levels of lipid oxidation ($p < 0.05$) compared to the control on days 1, 4 and 14 of storage (Table 6.2). In a recent *in vitro* study, heat treatment (85°C, 15 minutes) was reported to significantly improve the total flavonoid, tannin and sugar contents in addition to the antioxidant activities (DPPH, metal ion chelating ability, H_2O_2 scavenging, and FRAP) of three edible Irish brown seaweeds (*Laminaria saccharina*, *L. digitata* and *H. elongata*) (Rajauria et al., 2010). The spray-dried L/F extract examined in the present study is composed of polysaccharides which contain glucose. During cooking, reducing sugars such as glucose undergo non-enzymatic browning reactions such as the Maillard

reaction (Manzocco et al., 2000). The Maillard reaction has been associated with the formation of brown melanoidins (Maillard reaction products) with strong antioxidant capacity (Yilmaz & Toledo, 2005). The antioxidant activity of the L/F extract increased during cooking as demonstrated by the significant decrease in lipid oxidation of cooked pork patties, which may be attributed to Maillard reaction products formed during the cooking process which were not present in the fresh pork patties. Future studies are necessary to analyse the generation of Maillard reaction products during the cooking process.

6.3.3 Microbiology of fresh minced pork

In fresh pork patties, mesophilic and psychrotrophic total viable counts (TVC) increased during storage ranging from ~3.6 to 9.2 $\log_{10}$ cfu/g and ~5.0 to 9.8, respectively (Table 6.3). The TVC for 0.01% L/F, 0.1% L/F and 0.5% L/F were similar ($p > 0.05$) for all treatments, on each storage day, compared to controls indicating that L/F did not exert any antimicrobial activity in fresh pork patties. Similar findings were reported in Chapters 4 and 5 where supplementation of the L/F extract in porcine diets exhibited no effect on microbiological counts in fresh LTL muscle.

Results from the present study are in contrast to previously reported studies where antimicrobial activity of *L. digitata* was assessed using *in vitro* test systems such as the growth inhibition assay and agar plate diffusion test (Dubber & Harder, 2008). In related studies, dried *U. pinnatifida* (3%) and dried *H. elongata* (5%) added to beef patties and pork frankfurters, respectively, initially resulted in increased levels of microbial growth compared to controls, presumably due to the product formulations rather than the seaweed (i.e. lower salt concentrations). However trends were not statistically significant

and no antimicrobial properties of the seaweed were detected over time (López-López et al., 2009b; López-López et al., 2010). While many *in vitro* studies on the bioactive

Table 6.3. Effect of L/F extract addition on microbial status* (mesophilic and psychrotrophic) of fresh pork patties stored in modified atmosphere packs (80% O_2 : 20% CO_2) for up to 12 days at 4°C.

Treatment		**Storage time at 4°C, days**			
	Temp	**1**	**5**	**8**	**12**
Control	30°C (mesophilic count)	5.19 ± 0.08^{aA}	6.79 ± 0.37^{aB}	8.79 ± 0.59^{aC}	9.01 ± 0.46^{aC}
0.01% L/F		5.02 ± 0.28^{aA}	6.84 ± 0.45^{aB}	8.90 ± 0.61^{aC}	9.01 ± 0.62^{aC}
0.1% L/F		5.01 ± 0.24^{aA}	6.84 ± 0.23^{aB}	8.82 ± 0.62^{aC}	8.89 ± 0.74^{aC}
0.5% L/F		5.16 ± 0.06^{aA}	6.85 ± 0.22^{aB}	8.86 ± 0.69^{aC}	9.22 ± 0.58^{aC}
Control	4°C (psychrotrophic count)	6.30 ± 0.34^{aA}	8.17 ± 0.44^{aB}	9.32 ± 0.12^{aC}	9.46 ± 0.26^{aC}
0.01% L/F		6.26 ± 0.20^{aA}	8.16 ± 0.55^{aB}	9.26 ± 0.32^{aC}	9.31 ± 0.27^{aC}
0.1% L/F		6.37 ± 0.27^{aA}	8.19 ± 0.64^{aB}	9.48 ± 0.18^{aC}	9.23 ± 0.39^{aC}
0.5% L/F		6.37 ± 0.26^{aA}	8.33 ± 0.57^{aB}	9.63 ± 0.19^{aC}	9.75 ± 0.09^{aC}

[a]Within each parameter and storage day, mean values (± standard deviation) in the same column are not significantly different, $p > 0.05$.
[ABC]Within each treatment, mean values (± standard deviation) in the same row bearing different superscripts are significantly different, $p < 0.05$.
*$\log_{10}$CFU (colony forming units)/g pork.

compounds present in several seaweeds have demonstrated antimicrobial activity against a number of gram positive and negative bacteria, no scientific literature exists demonstrating antibacterial activity of seaweed extracts in food products (Gupta & Abu-Ghannam, 2011).

6.3.4 pH, water holding capacity and cook loss of fresh minced pork

The pH of fresh pork patties decreased from ~5.7 to 5.6 over the 14 day storage period and was unaffected by the addition of the L/F extract. This pH range is comparable to values reported previously (5.8-5.4) for post-mortem muscle (Faustman & Cassens, 1990). Choi et al., (2012) reported a significant decrease ($p < 0.05$) in the pH of reduced-fat pork patties when powdered *L. japonica* (1%, 3,% and 5%) was added due to the presence of acid components such as alginic acid (pH: 2.3-2.8) in the seaweed extract.

The addition of L/F to fresh pork patties had no significant effect on the water holding capacity (WHC) values which ranged from 32.3-37.8% and 31.5-34.6% on days 2 and 7 of storage, respectively (Table 6.4). Fleury and Lahaye (1991) demonstrated that the physicochemical properties of seaweeds are determined by the chemical structure of the constituent polysaccharides present in the algae. Previous studies on physicochemical properties of seaweeds report a positive correlation between water retention and swelling capacity (Rupérez & Saura-Calixto, 2001). Therefore meat products with added seaweed

Table 6.4. Effect of L/F extract addition on cook loss and water holding capacity (WHC) of fresh pork patties stored in modified atmosphere packs (80% O_2 : 20% CO_2) at 4°C.

Treatment	Storage time at 4°C, days			
	Cook Loss		WHC	
	2	7	2	7
Control	31.99 ± 0.93[a]	32.20 ± 0.94[a]	35.09 ± 1.72[a]	33.50 ± 3.37[a]
0.01% L/F	32.29 ± 1.23[a]	32.57 ± 0.58[a]	37.79 ± 1.62[a]	33.87 ± 2.83[a]
0.1% L/F	32.05 ± 0.31[a]	31.95 ± 1.53[a]	36.55 ± 1.82[a]	32.08 ± 1.67[a]
0.5% L/F	31.36 ± 1.53[a]	29.26 ± 1.31[a]	37.26 ± 1.40[a]	34.63 ± 3.60[a]
ST[*]	30.61 ± 0.77[a]	29.01 ± 4.00[a]	32.27 ± 4.10[a]	31.53 ± 4.72[a]

[a]Within each parameter and storage day, mean values (± standard deviation) in the same column are not significantly different, $p > 0.05$. [*]Salt control (ST), 0.005% salt.

or seaweed extracts, may have improved water and fat binding properties (López-López et al., 2009b).

Cook loss was unaffected by the addition of the L/F extract and ranged from 30.6-32.3% and 29.0-32.6% on days 2 and 7 of storage, respectively (Table 6.4). In a previous study, the cook loss of reduced-fat pork patties containing powdered *L. japonica* (1%, 3,% and 5%) was significantly lower than that of the control. Reduced cook loss was attributed to dietary fibres such as alginate and laminarin, which have high water holding and binding capacities (Choi et al., 2012). The functional properties of agar, alginates and carrageenans in food products have been well documented in the scientific literature. Scientific reports on laminarin and fucoidan focus primarily on biological activity (Elleuch et al., 2011; Thebaudin et al., 1997; Venugopal et al., 2008). The lack of effect

Table 6.5. Effect of L/F extract addition on texture profile analysis (TPA) of fresh pork patties stored in modified atmosphere packs (80% O_2 : 20% CO_2) at 4°C.

Treatment	Day	Hardness	Springiness	Cohesiveness	Gumminess	Chewiness
Control	2	36.56 ± 17.30[a]	0.86 ± 0.02[a]	0.55 ± 0.02[a]	20.12 ± 9.30[a]	17.12 ± 7.54[a]
0.01% L/F		38.68 ± 08.80[a]	0.85 ± 0.02[a]	0.55 ± 0.01[a]	21.37 ± 4.81[a]	18.14 ± 3.72[a]
0.1% L/F		52.34 ± 16.00[a]	0.86 ± 0.02[a]	0.55 ± 0.01[a]	28.55 ± 8.31[a]	24.54 ± 6.61[a]
0.5% L/F		44.20 ± 17.03[a]	0.84 ± 0.04[a]	0.56 ± 0.02[a]	24.33 ± 8.90[a]	20.26 ± 7.12[a]
Control	7	27.04 ± 06.62[a]	0.87 ± 0.02[a]	0.56 ± 0.02[a]	15.02 ± 4.03[a]	13.12 ± 3.84[a]
0.01% L/F		24.21 ± 06.67[a]	0.87 ± 0.04[a]	0.56 ± 0.01[a]	13.51 ± 3.48[a]	11.62 ± 2.66[a]
0.1% L/F		31.76 ± 10.50[a]	0.85 ± 0.04[a]	0.55 ± 0.02[a]	17.65 ± 5.94[a]	15.15 ± 5.32[a]
0.5% L/F		25.33 ± 02.03[a]	0.87 ± 0.03[a]	0.55 ± 0.02[a]	14.06 ± 1.21[a]	12.20 ± 1.16[a]

[a]Within each parameter and storage day, mean values (± standard deviation) in the same column are not significantly different, $p > 0.05$.

of the L/F extract in the present study may be due to the low concentration at which the extract was added to the fresh pork patties.

6.3.5 Texture profile analysis of fresh minced pork

General trends indicated that hardness increased with the addition of L/F in a dose-dependent manner on day 2 of storage compared to the controls, however results were not statistically ($p > 0.05$) significant (Table 6.5). In general, the addition of various types of fibre (soy, wheat, cereal or fruit) increased hardness of cooked meat emulsions (Fernández-Ginés et al., 2005). The hardness of gel/emulsion pork meat systems and frankfurters increased due to the addition of dried *H. elongata*, *U. pinnatifida*, and *P. umbilicalis* (2.5 and 5%), and *H. elongata* (5.6%), respectively (Cofrades et al., 2008; López-López et al., 2009a). The L/F extract did not have a significant effect on springiness, cohesiveness, gumminess or chewiness on either assessment day, relative to the controls (Table 6.5).

The proportion, composition (soluble and insoluble fractions) and characteristics (including particle size) of dietary fibre greatly influence physicochemical properties exerted in meat products (Gómez-Ordóñez et al., 2010). The texture of pork products may be enhanced by seaweed extracts depending on the amount and type of dietary fibre present in the extract. Conflicting studies demonstrated no beneficial effects of dietary fibre on pork texture (Cofrades et al., 2008; López-López et al., 2010). The thickening and gelling properties and the water-retention ability of soluble fibres contribute to the stabilization of the structure of foods (dispersions, emulsions and foams) by modifying rheological properties of the continuous phase. Insoluble fibres can also influence food texture due to their water-retention ability and swelling properties (Thebaudin et al., 1997). The direct addition of the L/F extract (composed of 17.1% soluble fibres) at low

levels did not significantly affect the textural parameters of the fresh pork patties in the present study which may be due to the very low level and type of soluble fibres added to the fresh pork patties.

6.3.6 Sensory evaluation of fresh and cooked minced pork

In 'visual' sensory analysis of minced pork patties (Table 6.6), the control and 0.01% L/F were highly significantly ($p < 0.01$) positively correlated to redness while 0.5% L/F was very highly significantly ($p < 0.001$) positively correlated to brownness on day 7 of storage. This is in agreement with instrumental '*a**' redness values, where a negative effect was exerted by L/F at the higher concentrations. Similarly, powdered *L. japonica* (1%, 3,% and 5%) was found to significantly ($p < 0.05$) decrease the colour score of reduced-fat pork patties due to the dark brown colour of the seaweed powder (Choi et al., 2012). On day 7, 0.01% L/F and the control were highly significantly ($p < 0.01$) positively correlated to purchasing appeal and overall acceptability while 0.5% L/F was very highly significantly ($p < 0.001$) negatively correlated to purchasing appeal and overall acceptability. Therefore, the 'visual' sensory properties of pork patties were negatively affected by the addition of 0.5% L/F extract, but at the lower level (0.01% L/F), were still acceptable to sensory panellists.

No significant trends were observed for 'eating quality' sensory analysis with the exception of tenderness, which was significantly ($p < 0.05$) negatively correlated with 0.5% L/F on day 7 of storage (Table 6.6). However, no significant effect on the TPA from the L/F extract was observed. This indicated that the L/F extract can be incorporated at low levels (0.01% - 0.1%) without detrimentally affecting the texture. No significant correlations were observed for overall acceptability and trends indicated positive directional correlations for control, 0.01% L/F and 0.1% L/F. In a previously

Table 6.6. Significance of regression coefficients (ANOVA values) for minced pork patties as derived by jack-knife uncertainty testing.

Parameter	**Storage time, days / Treatment**							
	Control		**0.01% L/F**		**0.1% L/F**		**0.5% L/F**	
Visual Sensory Analysis	**2**	**7**	**2**	**7**	**2**	**7**	**2**	**7**
Redness	-0.47	0.000***	0.28	0.003**	0.42	-0.06	-0.004**	-0.000***
Brownness	-0.51	-0.000***	-0.34	-0.002**	-0.45	0.06	0.08	0.000***
Drip	0.96	-0.24	0.97	-0.24	0.96	0.35	-0.96	0.21
Packaging quality	-0.76	0.05*	-0.78	0.05*	-0.77	-0.18	0.76	-0.03*
Purchasing appeal	0.57	0.000***	0.22	0.003**	0.28	-0.09	-0.02*	-0.000***
Overall acceptability	0.57	0.000***	0.25	0.002**	0.27	-0.08	-0.03*	-0.000***
Eating Quality Sensory Analysis								
Appearance	-0.82	0.49	-0.81	0.50	-0.81	-0.55	0.81	-0.47
Tenderness	-0.46	0.10	-0.72	0.09	-0.77	-0.30	0.68	-0.04*
Oxidation flavour	-0.55	0.15	-0.34	0.14	-0.39	-0.25	0.13	-0.14
Liking of flavour	0.80	-0.97	0.71	-0.97	0.60	0.97	-0.71	0.97
Overall acceptability	0.75	0.57	0.60	0.58	0.36	-0.63	-0.59	-0.56

Significance of regression coefficients; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

reported study, the addition of dried *U. pinnatifida* (3.3%) did not negatively affect the sensory properties of low-salt and low-fat beef patties in comparison with the control (López-López et al., 2010). Choi et al., (2012) reported the addition of powdered *L. japonica* (1%, 3% and 5%) to reduced-fat pork patties demonstrated significantly ($p < 0.05$) higher overall acceptability scores indicating foods containing seaweed had better sensory scores when compared to the controls. In the present study, sensory panellists were unable to distinguish between the control and pork patties containing low levels of the L/F extract.

6.4 CONCLUSIONS

Addition of a seaweed extract containing polysaccharides (soluble dietary fibres) did not enhance quality parameters of the fresh minced pork patties. The L/F extract at a level of 0.5% exerted a pro-oxidant effect on lipid oxidation over time attributed to the pro-oxidant components (sodium, copper and iron) present in the extract. Decreased lipid oxidation observed in cooked pork patties containing the L/F extract (0.5%) on days 1, 4 and 14, provided evidence that heating can enhance the antioxidant capacity of seaweed extracts in muscle foods and improve quality parameters possibly due to the formation of brown melanoidins (Maillard reaction products) with antioxidant functionality. The L/F extract at a level of 0.01% can be incorporated without adversely affecting the colour, lipid oxidation, texture or sensorial acceptance of pork patties. Further research is necessary to examine the effects of more refined or purified laminarin and fucoidan extracts in meat products.

CHAPTER 7

Seaweed polysaccharides (laminarin and fucoidan) as functional ingredients in pork meat: an evaluation of anti-oxidative potential, thermal stability and bioaccessibility

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ABSTRACT

The anti-oxidative potential of laminarin (L), fucoidan (F) and an L/F seaweed extract was measured using the DPPH free radical scavenging assay, in 25% pork (*longissimus thoracis et lumborum* (LTL)) homogenates (TBARS) (3 and 6 mg/ml) and in horse heart oxymyoglobin (OxyMb) (0.1 and 1 mg/ml). The DPPH activity of fresh and cooked minced LTL containing L (100 mg/g; L_{100}), F_{100} and $L/F_{100,\ 300}$, and bioaccessibility post *in vitro* digestion (L/F_{300}), was assessed. Theoretical cellular uptake of antioxidant compounds was measured in a transwell Caco-2 cell model. Laminarin displayed no activity and fucoidan reduced lipid oxidation but catalysed OxyMb oxidation. Fucoidan activity was lowered by cooking while the L/F extract displayed moderate thermal stability. A decrease in DPPH antioxidant activity of 44.15% and 36.63%, after 4 and 20 h respectively, indicated theoretical uptake of L/F antioxidant compounds. Results highlight the potential use of seaweed extracts as functional ingredients in pork.

7.1 INTRODUCTION

Seaweed polysaccharides (laminarin and fucoidan) isolated from the cell walls of brown seaweed (*Laminaria digitata*) possess immunomodulatory, anti-inflammatory, antiviral, antitumor, antithrombotic, anticoagulant and antioxidant bioactivities (Holdt & Kraan, 2011; Ngo et al., 2011). Structurally, laminarin is composed of β-(1,3)-linked glucose containing large amounts of sugars and a low fraction of uronic acids. Two types of polymeric chains are present in laminarin, G-chains with glucose at the end and M-chains with mannitol as the terminal reducing end (Devillé et al., 2004). The antioxidant activity of laminarin has been linked to molecular structure, degree and length of branching and the monosaccharide constituents (Choi et al., 2011). The structure of fucoidan consists mainly of α(1, 3)-linked L-fucopyranose residues with sulphates at the C-2 position (Anastyuk et al., 2012). Distinct conclusions regarding chemical structures of fucoidans are often difficult to formulate due to structural heterogeneity and lack of regularity in fucoidan molecules (Ustyuzhanina et al. 2014). Sulphate content, degree of sulphation and molecular weight are often attributed as factors influencing the antioxidant activity of fucoidan (Mak et al., 2013).

A wide range of analytical techniques (e.g. HPLC, ATR-FTIR and NMR spectroscopy) may be used to characterise and quantify structurally complex polysaccharides, such as laminarin and fucoidan, present in seaweeds (Kadam et al., 2014). Such techniques can involve detailed and time consuming extraction, preparation and sample clean-up procedures, depending on the parent seaweed material or the matrix in which the compounds of interest (polysaccharides) are contained (Gómez-Ordóñez et al., 2014). *In vitro* antioxidant assays (e.g. FRAP, ABTS, ORAC and DPPH free radical scavenging activities) are frequently used to assess the antioxidant activity and potency of plant extracts (Koleva et al., 2002). The DPPH assay (based on a quick electron transfer

reaction, followed by a slower hydrogen transfer reaction) is a simple, rapid, sensitive and reproducible index of antioxidant activity (MacDonald-Wicks et al., 2006). DPPH free radical scavenging activity of seaweed extracts, including laminarin and fucoidan, has been reported for a number of seaweed species (Machová & Bystrický, 2013; Mak et al., 2013).

The addition of antioxidant compounds to muscle foods (via the animals' diet or direct addition) in order to enhance meat quality and shelf-life has attracted much research attention in recent years. Previous research indicated that functional ingredients, such as laminarin and fucoidan, have beneficial effects pre- (animal health) (O'Doherty et al., 2010) and post-slaughter (meat quality) (Chapters 4 and 5). In Chapters 4 and 5, the addition of seaweed extracts, containing laminarin and fucoidan, to pig diets, resulted in lower levels of lipid oxidation in fresh pork steaks. However, direct addition of the same seaweed extract, promoted lipid oxidation and decreased the surface redness of fresh pork patties in Chapter 6. Catalysis of lipid oxidation was linked to the presence of salt and minerals in the seaweed extract. Increased discolouration (oxymyoglobin oxidation) was attributed to the effect of oxidising lipids and potential interactions between seaweed polysaccharides and oxymyoglobin. The anti- and pro-oxidative activity of laminarin and fucoidan on lipid and oxymyoglobin oxidation processes will be further examined in the present study.

The chemical structure of plant cell wall polysaccharides (e.g. cellulose, pectin substances, inulin and gums) and other associated non-carbohydrate components (i.e. resistant protein) can be sensitive to chemical, mechanical, thermal and enzymatic processing (Elleuch et al., 2011). Therefore the consequence of cooking on the potential bioactivity of laminarin and fucoidan in a meat matrix should be considered when formulating a functional meat product (Rawson et al., 2011). Cooking may sometimes

improve the antioxidant activity of plant based materials due to the formation of other antioxidant components such as Maillard reaction products (MRPs) (Gazzani et al., 1998). MRPs have been reported to possess antiradical activity including inhibition of the DPPH, oxygen peroxyl and hydroxyl radicals as well as copper and Fe^{2+} chelators (Gawlik-Dziki et al., 2009). In Chapter 6, a reduction in lipid oxidation of cooked minced pork patties containing laminarin and fucoidan which was attributed partially to the cooking process and the formation of MRPs which were not present in the fresh pork patties.

The digestion process may influence the bioactivity and bioaccessibility of laminarin and fucoidan. Bioaccessibility is defined as the fraction of a compound transferred from the food matrix during digestion, and thus made accessible for intestinal absorption and cellular uptake (Carbonell-Capella et al., 2014). *In vitro* digestion models provide a useful alternative to animal and human models and simulate the digestion process of the human gastrointestinal tract (GIT). Cell culture models, in particular the Caco-2 cell culture model, have been widely utilised as part of *in vitro* digestion models as a predictive tool for the absorption of bioactive compounds from foods (Hur et al., 2011).

Studies on the anti-oxidative potential of seaweed polysaccharides in meat products are limited and merit investigation. Furthermore, the literature lacks information regarding the bioaccessibility of seaweed polysaccharides in meat products after cooking and post digestion. The initial objective of this study was to profile the antioxidant activity of laminarin (L), fucoidan (F) and a seaweed extract containing L and F, using the DPPH free radical scavenging assay. The antioxidative potential of L, F and L/F was further examined in fresh pork *longissimus thoracis et lumborum* (LTL) homogenates and in commercial horse heart oxymyoglobin. The DPPH radical scavenging and thermal

stability of L, F and L/F in cooked pork patties was assessed. Finally cooked pork patties were subjected to an *in vitro* digestion procedure to determine the effects of digestion on the antioxidant potential of L, F and L/F and L/F digestates were examined in a transwell Caco-2 cell model to assess theoretical cellular uptake of antioxidant components of L/F.

7.2 MATERIALS AND METHODS

7.2.1 Reagents

All chemicals used were 'AnalaR' grade obtained from Sigma-Aldrich Ireland Ltd., Arklow, Co. Wicklow, Ireland and Merck KGaA, Darmstadt, Germany. Tissue culture plastics were supplied by Sarstedt, Wexford, Ireland and the Caco-2 cells were from the European Collection of Animal Cell Cultures, Wiltshire, UK. Fresh pork meat (*longissimus thoracis et lumborum* (LTL)) was supplied by Ballyburden Meat Processors, Ballincollig, Co. Cork, Ireland. Laminarin (L) (MW = 13 kDa) and fucoidan (F) (MW = 57 kDa) standards from Sigma-Aldrich were isolated from *Laminaria digitata* and *Fucus vesiculosus*, respectively. A spray-dried seaweed extract (L/F), containing laminarin and fucoidan was manufactured by Bioatlantis, Tralee, Co. Kerry, Ireland. The extract isolated from brown seaweed (*Laminaria digitata*) was prepared using an acid extraction technique, details of which are industrially-confidential. The extract composition is reported in Chapter 4.

7.2.2 Measurement of the DPPH free radical scavenging activities of seaweed polysaccharides (L, F and L/F)

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity of L, F and L/F was measured using the method of Qwele et al. (2013) with slight modifications. DPPH (0.2 mM, 3 ml) in methanol was added to 3 ml of L (1 and 10 mg/ml; L_1 and L_{10}), F (1 mg/ml; F_1) and L/F (1 and 3 mg/ml; L/F_1 and L/F_3). Trolox C (1 mg/ml; Trolox), was used as a positive control. Tubes were mixed and incubated for up to 20 h at room temperature (~20°C) in the dark. The assay control contained 3 ml distilled water and 3 ml of DPPH solution. Absorbance measurements were recorded spectrophotometrically (Cary 300 Bio, UV-Vis spectrophotometer, Varian Instruments,

Palo Alto, CA, USA) against a distilled water blank after 1, 4 and 20 h at 517 nm. The DPPH free radical scavenging activity, expressed as a percentage of the assay control was calculated as follows:

% inhibition of DPPH = [1− (absorbance of sample / absorbance of assay control)] x 100

7.2.3 The effect of seaweed polysaccharides on lipid oxidation in pork muscle homogenates

Pork homogenates (25% w/v) were prepared by homogenising LTL (70 g) in buffer (210 ml) (0.12 M KCL 5 mM histidine, pH 5.5) on ice using an Ultra-turrax T25 homogeniser. L, F and L/F were solubilised in distilled water and added to LTL homogenates at final concentrations of 3 and 6 mg/ml (L_3, L_6, F_3, F_6, L/F_3 and L/F_6) homogenate. Lipid oxidation in muscle homogenate samples (20 g) held at 4°C was initiated by the addition of 45 µM $FeCl_3$/sodium ascorbate (1:1). Muscle homogenates with and without $FeCl_3$/sodium ascorbate and without antioxidants (L, F and L/F) were run simultaneously as controls with each experiment. Lipid oxidation measurements were measured after 4 h in samples held at 4°C.

7.2.3.1 Measurement of lipid oxidation in pork muscle homogenates

A modification of the 2-thiobarbituric acid (TBA) assay of Siu & Draper (1978) was used to measure lipid oxidation in pork muscle (LTL) homogenates. Homogenate samples (4 ml) were added to 4 ml 10% trichloroacetic acid (TCA) and centrifuged (Beckman J2-21, Beckman Instruments Inc., Brea, CA, USA) at 6160g for 15 min at 4°C. Following centrifugation, the supernatant was filtered through Whatman No. 1 filter paper. In a screw cap test tube, the clear filtrate (4 ml) was added to 0.06 M TBA reagent (1 ml) and incubated at 80°C for 90 min. The absorbance of the resulting coloured

complex was measured using a spectrophotometer (Cary 300 Bio) at 532 nm against a blank containing buffer (2 ml, 0.12 M KCL 5 mM histidine, pH 5.5), 10% TCA (2 ml) and 0.06 M TBA reagent (1 ml). Results were expressed directly as absorbance values at 532 nm.

7.2.4 The effect of seaweed polysaccharides on oxymyoglobin oxidation

7.2.4.1 Preparation of commercial oxymyoglobin

Commercial horse heart oxymyoglobin (OxyMb) was prepared according to a modification of the method of Brown & Mebine (1969). Metmyoglobin (MetMb) (0.06 g) was dissolved in ice-cold distilled water (2 ml) to a concentration of 30 mg/ml and reduced to OxyMb by the addition of sodium dithionite at 1 mg/ml. To remove excess dithionite, OxyMb solution (2 ml) was applied to a glass column (2 cm i.d x 25 cm) containing 10 g of mixed bed ion exchange resin (Amberlite MB-1A) and eluted from the column with approximately 20 ml cold distilled water. The OxyMb solution was passed through the column three times to reduce the conductivity to that of distilled water and was adjusted to a final volume of 50 ml with double strength buffer (300 mM KH_2PO_4-KOH, pH 5.5). The concentration of OxyMb in the final solution was calculated from its absorbance value at 525 nm divided by a millimolar extinction coefficient of 7.6 $mM^{-1}cm^{-1}$ (Krzywicki, 1982).

7.2.4.2 Effect of seaweed polysaccharides on oxymyoglobin oxidation

Incubates (7 ml) containing OxyMb (~1 mg/ml) and L, F and L/F at two levels (0.1 and 1 mg/ml; $L_{0.1}$, L_1, $F_{0.1}$, F_1, $L/F_{0.1}$ and L/F_1) in 150mM KH_2PO_4-KOH, pH 5.5, were prepared. Distilled water was used to prepare seaweed polysaccharide solutions (20 mg/ml). Additions to each OxyMb incubate were at a final concentration of 5% (v/v).

Incubates were held at 4°C and OxyMb oxidation was measured on days 0, 4 and 8 of storage.

Following centrifugation at 6160g for 10 min at 4°C, the absorbance spectra of the incubates (2 ml) containing commercial OxyMb were measured on a spectrophotometer (Cary 300 Bio) and spectral scans were recorded from 750 to 500 nm. The relative proportion of OxyMb (% of total myoglobin) was calculated using absorbance measurements at selected wavelengths (525, 545, 565, 572 and 730 nm (turbidity)) and the following equation:

$$\text{OxyMb, \%} = (0.882R_1 - 1.267R_2 + 0.809R_3 - 0.361) \times 100$$

where R_1, R_2 and R_3 are the absorbance ratios A^{572}/A^{525}, A^{565}/A^{525} and A^{545}/A^{525}, respectively (Krzywicki, 1982).

7.2.5 Effect of cooking on DPPH free radical scavenging activity of seaweed polysaccharides in pork meat

Fresh minced LTL was assigned to one of five treatments: untreated pork (Control), L (100 mg/g pork; L_{100}), F (100 mg/g; F_{100}), L/F (100 mg/g; L/F_{100}) and L/F (300 mg/g; L/F_{300}). The levels of L, F and L/F added to fresh minced LTL were based on the DPPH free radical scavenging activities of the seaweed polysaccharides determined in section 7.2.2. L, F and L/F were dissolved in water, immediately added to fresh minced LTL (5% v/w) and mixed vigorously. Minced LTL (1 g portion) from each treatment was retained for measurement of DPPH free radical scavenging activity of fresh minced LTL prior to cooking. The remaining fresh LTL (5 g portions) of each treatment were placed on aluminium foil lined trays and cooked at 180°C for 5 min 30 sec in a fan-assisted convection oven (Zanussi Professional, Model 10 GN1/1, Conegliano, Italy) until an internal temperature of 72°C was reached.

Fresh and cooked minced LTL (1 g) were homogenised in 0.05 M phosphate buffer (9 ml), pH 7, using an Ultra Turrax T25 homogeniser and homogenates were centrifuged (Beckman J2-21, Beckman Instruments Inc., CA, USA) at 7,800g for 10 min at 4°C. The supernatant fraction obtained (fresh/cooked minced LTL) was used for the measurement of the DPPH free radical scavenging activity (Qwele et al., 2013). DPPH (0.2 mM, 3 ml) prepared in methanol was added to 0.3 ml supernatant and 2.7 ml distilled water. The mixture was vortexed and left to stand at room temperature (~20°C) in the dark. The assay control contained 0.3 ml phosphate buffer and 2.7 ml distilled water and 3 ml of DPPH solution. The absorbance of the solution was measured against a distilled water blank after 1, 4 and 20 h at 517 nm. The scavenging activity of the pork meat against the DPPH radical before and after cooking was expressed as a percentage of the assay control and calculated as:

% inhibition of DPPH = [1− (absorbance of sample / absorbance of assay control)] x 100

7.2.6 Effect of *in vitro* digestion on the DPPH free radical scavenging activity of seaweed polysaccharides in cooked pork meat

The *in vitro* digestion procedure was adapted from that previously described by Daly et al. (2010). All experimental work was carried out in UV-light free conditions to reduce the possible photo-decomposition of L, F and L/F present in the cooked minced LTL. Briefly, cooked minced LTL (1 g) from each treatment were weighed into 100 ml plastic tubes and homogenized using an Ultra Turrax T25 homogeniser at 24,000 rpm for 10 sec in 8 ml Hanks Balance Salts Solution (HBSS) containing BHT. HBSS (5 ml) was slowly pipetted down the homogeniser to rinse remaining residue into the plastic tubes. The homogenates were transferred into amber bottles (rinsed twice using 5 ml HBSS). In order to mimic the gastric phase of digestion, pepsin (1 ml) (0.04 g/ml in 0.1 N HCl) and

HBSS (2 ml) was added and the pH was adjusted to 2 using 1 M HCl. Oxygen was displaced by blowing nitrogen over the samples for 5 sec. Samples were then incubated at 37°C for 1 h in an orbital shaking (95 rpm) water bath (Grant OLS200, Keison Products; Essex, UK).

After 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). Subsequently, the pH was increased 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 h. Following intestinal digestion, the digested minced LTL (digestates) from each treatment were centrifuged (Beckman J2-21) at 7,800 g for 10 min at 4°C. Undigested minced LTL samples were diluted using HBSS to the same final volume as the digestates and subsequently centrifuged at 7,800 g for 10 min at 4°C.

The supernatant (aqueous fractions) of the undigested minced LTL and digestate samples were frozen at -80°C until required for measurement of DPPH free radical scavenging activity (described in section 7.2.5). The assay control contained 0.3 ml HBSS buffer and 2.7 ml distilled water and 3 ml of DPPH solution. The absorbance of the solution was measured against a distilled water blank after 1, 4 and 20 h at 517 nm. The scavenging activity of the pork meat against DPPH radical post digestion was corrected for the meat control and expressed as:

$$\% \text{ inhibition of DPPH} = [(1- (Ab_{sample} / Ab_{ac})) \times 100] - [(1- (Ab_{meatcontrol} / Ab_{ac})) \times 100]$$

Where Ab_{sample} = absorbance of sample; Ab_{ac} = absorbance of assay control; $Ab_{meatcontrol}$ = absorbance of meat control.

7.2.7 Uptake and transport of the aqueous fraction of digested minced LTL

Caco-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), containing 10% (v/v) foetal bovine serum (FBS) and 1% (v/v) non-essential amino acids. Cells were grown at 37°C /5% CO_2 in a humidified incubator and were cultured with 0.5% Penicillin-Streptomycin (5,000 U/ml). Cultures of Caco-2 cells were used between passages 46-51. To establish the Caco-2 intestinal model, the cells were seeded at a density of 6 x 10^4 cells cm^{-2} on a transwell plate (12-well plate, 22 mm diameter, 0.4 µm pore size membrane). Media was changed every 2-3 days and experiments were performed when monolayers were 17-20 days post-confluency. The aqueous fraction of the digestates (control and L/F_{300}) (125 µl) were diluted to a final volume of 500 µl with serum free media and added to the top chamber of the transwell plate. Serum free media (1 ml) was added to the basolateral chamber and the cells were incubated for 4 and 20 h. Preliminary work showed that the aqueous fraction of the digestates was not toxic to the cells (data not shown). The transepithelial electrical resistance (Millicell-ERS, Millipore, Cork, Ireland) was measured before and after the addition of the aqueous fraction of the digestates to ensure the monolayer remained intact. The media from the basolateral chamber was then harvested for the measurement of the DPPH free radical scavenging activity (see section 7.2.5) after transepithelial transport and cellular uptake.

The assay control contained 0.3 ml serum free media and 2.7 ml distilled water and 3 ml of DPPH solution. The absorbance of the solution was measured against a distilled water blank after 4 h at 517 nm. The difference between the DPPH free radical scavenging activities of L/F_{300} and the control, expressed as a percentage of the control, was calculated for the aqueous fraction of the digestate (AF) and the transwell basolateral chamber media (TW) as follows:

% theoretical transepithelial transport and cellular uptake = [($AF_{L/F300}$ - $AF_{meatcontrol}$) / $AF_{meatcontrol}$) x 100] - [($TW_{L/F300}$ - $TW_{meatcontrol}$) / $TW_{meatcontrol}$) x 100]

Where $AF_{L/F300}$ = absorbance of aqueous fraction of the digestate L/F_{300}; $AF_{meatcontrol}$ = absorbance of aqueous fraction of the digestate meat control; $TW_{L/F300}$ = absorbance of transwell basolateral chamber media following cellular uptake of L/F_{300}; $TW_{meatcontrol}$ = absorbance of transwell basolateral chamber media following cellular uptake of the meat control. The difference in activity between AF and TW was attributed to uptake of antioxidant compounds by the Caco-2 cells.

7.2.8 Statistical analysis

Each experiment was carried out three individual times. All analyses were performed in duplicate. DPPH free radical scavenging of L, F and L/F, lipid oxidation and oxymyoglobin oxidation means were analysed by one-way ANOVA. Means were considered significantly different at ($p < 0.05$) using Tukey's post hoc test. Following cooking, digestion and cellular uptake, mean sample values ($n = 3$) were subjected to a full repeated measures two-way analysis of variance (ANOVA) to investigate the effects of L, F and L/F addition, time and their interactions. L, F and L/F represented the 'between-subjects' factor and the effect of time was measured using the 'within-subjects' factor. Tukey's test was used to adjust for multiple comparisons between treatment means. All analysis was carried out using the SPSS 18.0 for Windows (SPSS, Chicago, IL, USA) software package.

7.3. RESULTS AND DISCUSSION

7.3.1 Free radical scavenging activity of seaweed polysaccharides (L, F and L/F)

In general, the DPPH free radical scavenging activity of seaweed polysaccharides increased over 20 h and followed the order: Trolox > F_1 > L/F_3 > L/F_1 > L_{10} ≈ L_1 (Table 7.1). DPPH free radical scavenging activity of L/F increased as a function of concentration. The DPPH free radical scavenging activities reported for L_1 and L_{10} were comparable to previously reported values (1.4-5.3%) for laminarin extracted from *Laminaria digitata* at concentrations ranging from 0.125 to 1.0 mg/ml (Machová & Bystrický, 2013). The DPPH free radical scavenging activity of F_1 (66.13%) after 1 h in the present study was similar to the inhibition of the DPPH radical (55.22%) after 30 minutes by fucoidan (1 mg/ml) from Sigma reported by Mak et al. (2013).

Table 7.1. Free radical scavenging activity (DPPH) of L, F and L/F for up to 20 h at ~ 20°C.

Incubate	**time, hours**		
	1	**4**	**20**
L_{1*}	1.09 ± 0.92^{ab}	1.39 ± 0.96^{ab}	1.64 ± 1.30^{ab}
L_{10}	1.55 ± 1.21^{b}	2.72 ± 1.77^{b}	3.16 ± 2.72^{b}
F_1	66.13 ± 0.32^{c}	76.48 ± 0.30^{c}	90.68 ± 0.55^{c}
L/F_1	35.43 ± 2.04^{d}	47.35 ± 1.79^{d}	69.51 ± 1.37^{d}
L/F_3	56.18 ± 1.01^{e}	68.40 ± 0.89^{e}	78.41 ± 0.21^{e}
Trolox	95.89 ± 0.08^{f}	95.92 ± 0.14^{f}	95.76 ± 0.48^{f}

*Subscripts 1, 3 and 10 denote concentrations in mg/ml.
abcdefWithin each storage time, mean values (± standard deviation) in the same column bearing different superscripts are significantly different, $p < 0.05$.

Limited research suggests that carbohydrate polymers such as β-glucans (laminarin) possess free radical scavenging activity, however the addition of high levels of β-glucans is often necessary before radical scavenging activity is observed (Mirjana et al., 2013; Tsiapali et al., 2001). At concentrations of 20-200 mg/ml (higher than those used in the present study) a 1,3 β-D-glucan enriched extract from cereal grains demonstrated 25-

80% inhibition of the DPPH radical (Mirjana et al., 2013). The mechanism of antioxidant action of β-D-glucans against free radicals is still not well understood, but a number of theories exist (Giese et al., 2014). Tsiapali et al. (2001) reported enhanced antioxidant activity of laminarin polymers over monomeric units due to greater ease of abstraction of anomeric hydrogen from one of the internal monosaccharide units rather than from the reducing end. In the present study, laminarin exhibited weak radical scavenging activity which may be due to the level examined.

For some antioxidants, such as Trolox, the reaction with DPPH is rapid while other compounds may react more slowly (Huang et al., 2005). The ability of seaweed extracts to quench free radicals is known to take place over longer periods of time compared to rapid acting synthetic antioxidants such as butylated hydroxyanisole (BHA) (Abu-Ghannam & Cox, 2014; Yuan et al., 2005). Slower reacting compounds are hypothesised to have a more complex reaction mechanism involving one or more secondary reactions in quenching the DPPH radical (Koleva et al., 2002). In the present study, after 20 h, the DPPH free radical scavenging activity of F_1 was equivalent (although statistically lower) to the positive control (Trolox), and significantly ($p < 0.05$) higher than both L_1 and L_{10}. Therefore the ability of an antioxidant to reduce and quench free radicals over a longer period of time may have benefits for extending the shelf-life of processed foods (Yuan et al., 2005).

7.3.2 Effect of seaweed polysaccharides on lipid oxidation in pork muscle model systems

In vitro antioxidant assays (e.g. the DPPH assay) highlight the potential antioxidant activities of compounds but may not accurately predict activity in complex test systems such as muscle foods. To further investigate antioxidant activities of L, F

and L/F, pork meat homogenates were subjected to iron/ascorbate ($FeCl_3$/sodium ascorbate)-induced lipid oxidation. Compared to the control, after 4 h at 4°C, lipid oxidation significantly increased ($p < 0.05$) in the pork meat homogenates with the addition of pro-oxidants (Figure 7.1). No difference was observed for L_3 and L_6 compared to the control. Similarly no inhibition of lipid oxidation by laminarin, at levels comparable to those in the present study (3 mg/ml), was observed in a linoleic acid emulsion system (Giese et al., 2014). F_3 and F_6 significantly decreased ($p < 0.05$) levels of lipid oxidation in pork meat homogenates. Trends indicated that levels of lipid oxidation in L/F_3 and L/F_6 were lower than the control (with pro-oxidants) although results were not statistically significant. In Chapter 6, salt and minerals, present in the L/F extract, may have promoted lipid oxidation in fresh pork patties. Minerals and salt present in L/F_3 and L/F_6 may have counteracted the antioxidant activity of other constituents in the extract, thus impeding ability to significantly enhance lipid stability in the pork meat homogentates (Figure 7.1).

Structurally laminarin does not contain sulphate groups, which reportedly increases the antioxidant activity of fucoidan (Zhou et al., 2014). Sulphate groups can enhance the steric hindrance between polymer chains in polysaccharides leading to a more ordered and expanded conformation thus improving homogeneity in aqueous solution (Yan et al., 2012). Lower molecular weight polysaccharides are often linked to increased free radical scavenging ability, presumably due to a non-compact structure which may allow more available sulphate and hydroxyl groups react with free radials (Gómez-Ordóñez et al., 2014). However, this was not observed for L in the present study indicating that even at low molecular weight, the structure in the presence of pork meat was unable to inhibit lipid oxidation, similar to the lack of DPPH free radical scavenging activity observed in section 7.3.1 (Table 7.1).

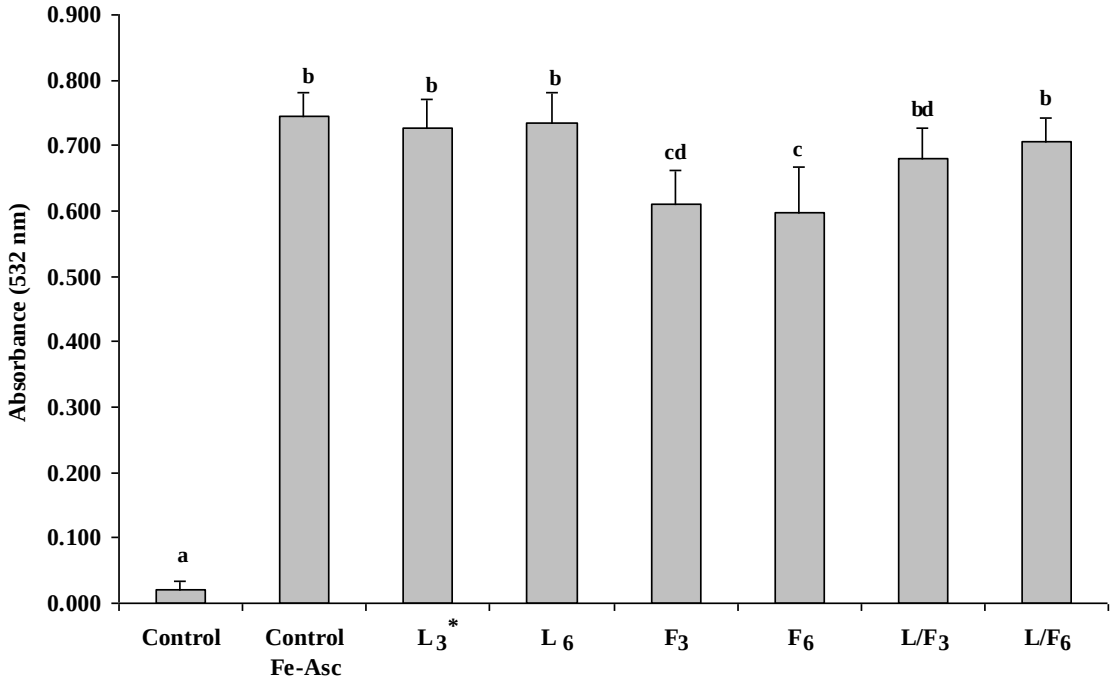

Figure 7.1. Lipid oxidation in 25% *longissimus thoracis et lumborum* (LTL) pork muscle homogenates following the addition of L, F or L/F and storage for up to 4 h at 4°C. * Subscripts 3 and 6 denote concentrations in mg/ml. [abcd]Mean values (± standard deviation error bars) bearing different superscripts are significantly different, $p < 0.05$.

In general, it is accepted that natural antioxidants scavenge free oxygen-centered radicals via two major mechanisms, hydrogen atom transfer (HAT) reactions and electron transfer (ET) reactions. Yan et al. (2012) suggested the HAT reaction is more likely to occur in neutral polysaccharides, such as laminarin, while the ET is the probable mechanism in acidic polysaccharides, like fucoidan where the negative charge of the sulphate groups plays a large part in the radical scavenging activity. In the present study, fucoidan is most likely responsible for the antioxidant activity observed by the L/F extract in the pork meat homogenates presumably due to ET reactions between the sulphate groups and the free radicals in the pork meat homogenates.

7.3.3 Effect of seaweed polysaccharides on oxymyoglobin oxidation

Oxymyoglobin oxidation (represented by a reduction in OxyMb, %) increased during storage for up to 8 days at 4°C (Table 7.2). $L_{0.1}$ and L_1 had no influence on OxyMb oxidation, however $F_{0.1}$ and F_1 significantly ($p < 0.05$) enhanced OxyMb oxidation compared to the control in a dose dependant manner. Similarly, a significant increase ($p < 0.05$) in OxyMb oxidation was observed for $L/F_{0.1}$ and L/F_1. The presence of metmyoglobin is characterised by an increased absorption at ~628 nm (Schenkman et al., 1997) which is evident in the spectral scan for OxyMb alone and OxyMb + F_1 (Figure 7.2). At the wavelengths examined, no spectral shift in the presence of F_1 was observed.

Table 7.2. Oxymyoglobin (OxyMb) oxidation (represented by a reduction in OxyMb) following the addition of L, F or L/F and storage for up to 8 days at 4°C.

Incubate	**time, days**		
	0	**4**	**8**
Control	76.53 ± 2.28^{a}	59.92 ± 2.30^{a}	54.46 ± 2.02^{a}
$L_{0.1}$	76.57 ± 2.31^{a}	59.68 ± 2.14^{ab}	54.00 ± 2.50^{a}
L_1	76.59 ± 2.73^{a}	58.11 ± 3.12^{abc}	52.51 ± 2.75^{a}
$F_{0.1}$	74.73 ± 2.54^{ac}	53.44 ± 2.44^{bd}	45.42 ± 2.56^{b}
F_1	67.55 ± 2.50^{b}	32.95 ± 2.00^{e}	21.71 ± 1.34^{c}
$L/F_{0.1}$	74.93 ± 2.06^{ad}	52.91 ± 2.44^{cd}	44.95 ± 2.82^{b}
L/F_1	69.03 ± 2.78^{bcd}	39.01 ± 1.90^{e}	28.78 ± 2.25^{d}

*Subscripts 0.1 and 1 denote concentrations in mg/ml.
[abcde]Within each storage time, mean values (± standard deviation) in the same column bearing different superscripts are significantly different, $p < 0.05$.

The exact mechanism by which fucoidan promotes OxyMb oxidation is unclear. The ability of fucoidan to bind to proteins such as antithrombin (a glycoprotein) and bovine serum albumin (a globular protein) has previously been linked to molecular weight as well as the sulphation patterns of the polysaccharide (Kim & Shin, 2015; Mulloy, 2005; Varenne et al., 2003). Generally, interactions between anionic polysaccharides and positively charged OxyMb have been reported to be electrostatic in

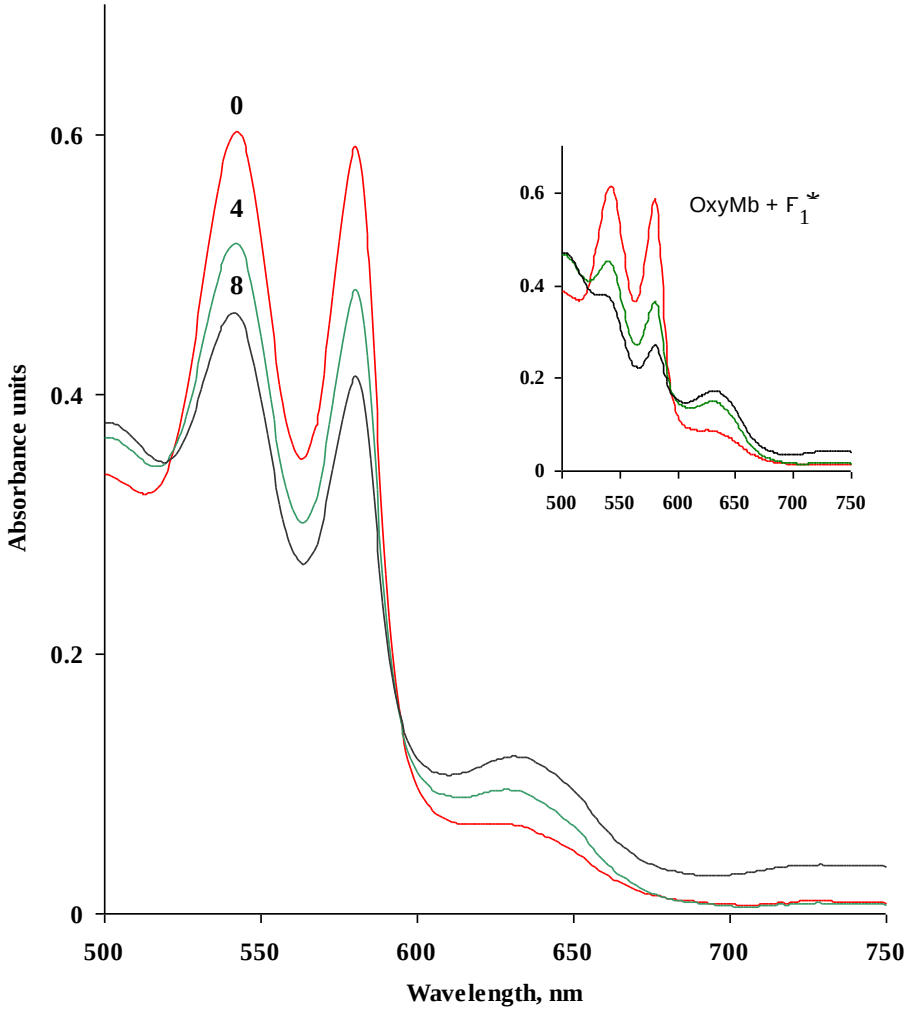

Figure 7.2. Absorbance spectra of oxymyoglobin (OxyMb) alone and following the addition of F_1 (*Subscript 1 denotes concentration in mg/ml) and storage for up to 8 d at 4°C.

nature due to opposing charges (Imeson et al., 1977). Similarly, Satoh & Shikama (1981) demonstrated that oxidation of OxyMb was initiated via nucleophilic attack at the iron (II) centre of OxyMb by a water molecule with strong proton assistance from the distal histidine, or a hydroxide anion (OH^-). These reactions can cause irreversible displacement of bound dioxygen from OxyMb resulting in the formation of ferric metmyoglobin and generation of the superoxide anion. In the present study, the anionic sulphate groups of fucoidan potentially enhanced the oxidation of OxyMb through the nucleophilic displacement mechanism described above.

7.3.4 Effect of cooking on the DPPH free radical scavenging activity of seaweed polysaccharides in pork meat

Statistical analysis indicated that the DPPH free radical scavenging of L, F and L/F in the presence of fresh minced LTL ($F_{100} > L/F_{300} \approx L/F_{100} \approx L_{100}$) followed a similar pattern to the DPPH free radical scavenging activities of the seaweed polysaccharides reported in section 7.3.1. L_{100} DPPH free radical scavenging was similar to the control before and after cooking (Figure 7.3). The DPPH free radical scavenging activity of F_{100} significantly ($p < 0.05$) decreased after cooking. Thermal processing is known to modify the physicochemical properties of plant cell wall polysaccharides (Elleuch et al., 2011). The DPPH free radical scavenging activities of fresh and cooked L/F_{100} and L/F_{300} were similar indicating moderate thermal stability of the L/F extract. Similarly, in Chapter 5, low to moderate thermal stability of L/F in cooked minced pork patties from pigs fed the L/F extract for 3 weeks pre-slaughter was observed.

L/F_{300} significantly ($p < 0.05$) enhanced the DPPH free radical scavenging activity of cooked minced LTL compared to the control (Figure 7.3). Similarly, Prabhasankar et al. (2009) reported an increase in DPPH free radical scavenging activity of cooked pasta with the addition of a brown seaweed (*Undaria pinnatifida*) to uncooked pasta. The formation of Maillard reaction products (MRP) and other novel antioxidant compounds such as mycosporine-like amino acids during heat treatment of seaweed extracts has been reported (Kuda et al., 2006; Rajauria et al., 2010; Yoshiki et al., 2009). Additionally, MRP have proven effective inhibitors of lipid oxidation in cooked minced pork patties (Bedinghaus & Ockerman, 1995). In the present study, MRP formed during heating of L/F_{300} most likely enhanced the DPPH free radical scavenging of cooked minced LTL.

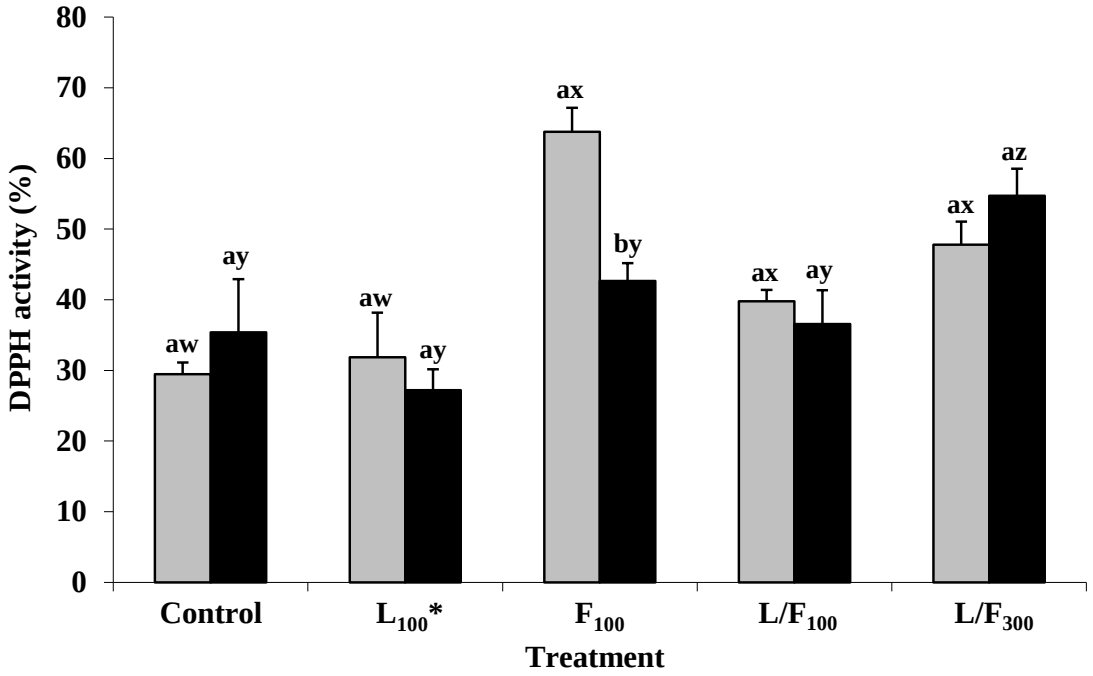

Figure 7.3. Free radical scavenging activity (DPPH) of L, F or L/F in fresh and cooked minced *longissimus thoracis et lumborum* (LTL) pork muscle stored for 20 h at ~20°C. *Subscripts 100 and 300 denote concentrations in mg/g. [ab]Within each treatment, mean values (± standard deviation error bars) bearing different superscripts are significantly different, $p < 0.05$. Comparing [wx] fresh and [yz] cooked LTL pork muscle treatments to their respective controls, mean values bearing different superscripts are significantly different, $p < 0.05$. (□), fresh; (■), cooked.

7.3.5 DPPH free radical scavenging activity of seaweed polysaccharides in pork meat following *in vitro* digestion

During the digestion procedure, cooked minced LTL from each treatment was subjected to pH changes and enzymatic reactions which resulted in increased (~30-44%) DPPH free radical scavenging activities in digestates compared to undigested aqueous fractions (data not shown). The DPPH free radical scavenging activity of the control post digestion increased from 14.4% to 44.8% and was attributed to the presence of compounds such as peptides released from the pork meat during the *in vitro* digestion procedure. Escudero et al. (2010) reported 51 different peptides were released from pork meat (*longissimus dorsi*) following *in vitro* digestion. Additionally, peptides obtained from animal sources such as porcine myofibrillar proteins have demonstrated DPPH free

radical scavenging activity (Chen et al., 1998; Saiga et al., 2003; Sarmadi & Ismail, 2010). Data from each treatment (L_{100}, F_{100}, L/F_{100} and L/F_{300}) were adjusted for the meat control to estimate the antioxidant activity due to the seaweed polysaccharides post digestion (Figure 7.4).

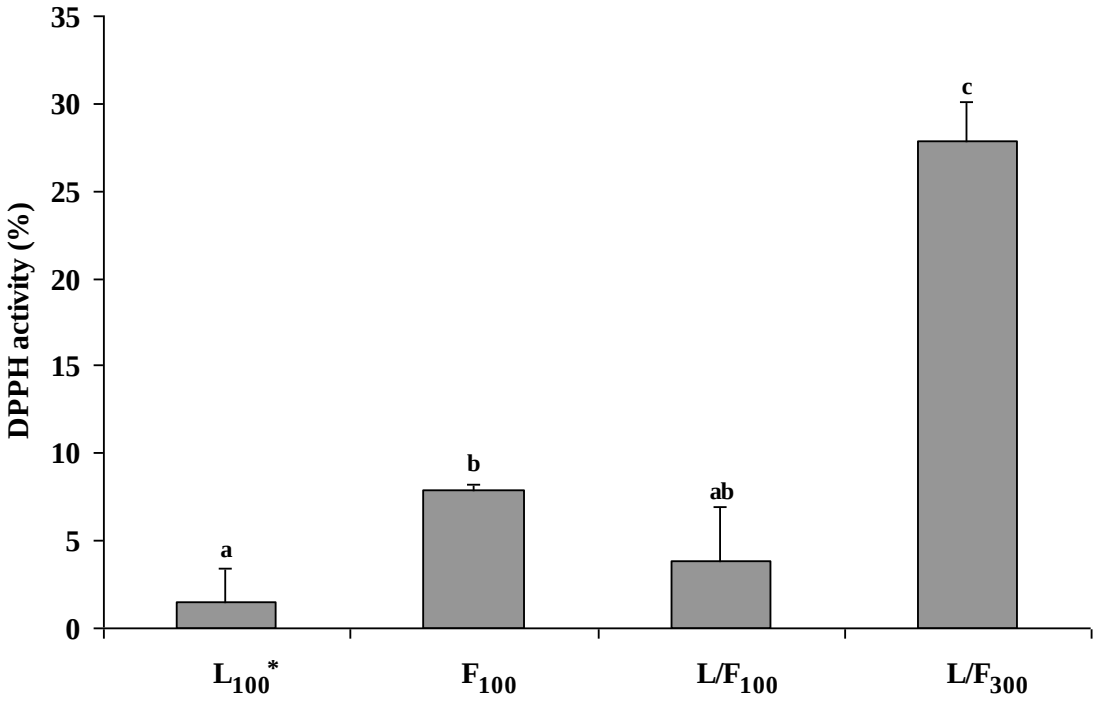

Figure 7.4. Free radical scavenging activity (DPPH) of L, F or L/F in digested cooked minced *longissimus thoracis et lumborum* (LTL) pork muscle stored for 20 h at ~20°C and adjusted for the control. *Subscripts 100 and 300 denote concentrations in mg/g. abcMean values (± standard deviation error bars) bearing different superscripts are significantly different, $p < 0.05$.

The DPPH free radical scavenging activity of digested L_{100} and L/F_{100} were similar (Figure 7.4). Laminarin is resistant to digestion in the upper GIT including acidic and enzymatic hydrolysis (O'Sullivan et al., 2010). Salyers et al. (1977) established two different types of enzymes (laminarases and β-glucosidases) were essential to fully degrade laminarin and were only synthesised after 4-6 h of incubation in the presence of the inducer. In the present study, the lack of suitable enzymes to break down laminarin in

the *in vitro* digestion model used may explain the lack of enhanced antioxidant activity post digestion.

F_{100} and L/F_{300} significantly ($p < 0.05$) enhanced the DPPH free radical scavenging activity of cooked minced LTL post digestion. A few fucan-degrading enzymes have been obtained from marine bacteria and molluscs, however complete enzymatic breakdown has not been reported. The presence of sulphate groups attached to fucoidan has been postulated as a reason for resistance to enzymatic breakdown during digestion. The retention of the sulphate groups during digestion results in high ionic exchange capacities such as the binding of bile salts and scavenging of free radicals throughout the GIT before potential absorption (Michel & Macfarlane, 1996). The enhanced DPPH radical scavenging activity of F_{100} and L/F_{300} in cooked minced LTL, in the present study, may be due to the retention of the sulphate groups throughout the *in vitro* digestion procedure.

The DPPH free radical scavenging activity of digested L/F_{300} was significantly ($p < 0.05$) greater than F_{100}. Fucoidan may be partially responsible for the scavenging activity of the extract. The synergistic effect between components in the L/F extract, such as protein and mannitol, could have contributed to the observed enhanced free radical scavenging activity in cooked minced LTL post digestion. Antioxidant activity post-digestion of bioactive peptides extracted from seaweeds has been reported previously (Kim et al., 2007). Mannitol is frequently considered as a reference for carbohydrate-type antioxidants due to its established scavenging abilities (Tsiapali et al., 2001). Additionally, MRPs formed during cooking may have enhanced the DPPH free radical scavenging activity of L/F_{300} post digestion.

7.3.6 Bioaccessibility of seaweed polysaccharides in pork meat after incubation with Caco-2 cells

The aqueous fraction of the control and L/F_{300} digestates was incubated with Caco-2 cells for 4 and 20 h to determine the bioaccessibility of L/F post-digestion. The DPPH free radical scavenging activity of L/F_{300}, post digestion, was 56.49% higher than the meat control. Following incubation of the control and L/F_{300} digestates with Caco-2 cells for 4 and 20 h, the DPPH free radical scavenging activity of L/F_{300} was 12.34% and 19.85% higher than the meat control, respectively. The reduction in the DPPH free radical scavenging activity indicated theoretical uptake of some compounds with antioxidant activity. Therefore theoretical cellular uptake of seaweed polysaccharides was 44.15% and 36.63% (DPPH free radical scavenging activity) after incubation with Caco-2 cells at 4 and 20 h, respectively. Similarly, Soler-Rivas et al. (2009) reported a decrease in ABTS free radical activity after digested grilled mushrooms were incubated with Caco-2 cells indicating absorption of antioxidant compounds. Previously reported studies indicated that seaweed polysaccharides can be, to some extent, absorbed into the blood stream post digestion; however metabolism of these components after absorption has not been established (Holdt & Kraan, 2011). Antioxidant compounds from L/F_{300} not absorbed through the intestinal wall would potentially be able available to scavenge free radicals or be fermented by colonic bacteria and contribute to the overall antioxidant defence system of the GIT (Holdt & Kraan, 2011; Palafox-Carlos et al., 2011). Further research is necessary to determine the fate of antioxidant compounds after absorption.

7.4. CONCLUSIONS

Due to the presence of sulphate groups and anionic charge, fucoidan is a more potent free radical scavenging antioxidant than laminarin. Furthermore fucoidan is at least, in part, responsible for the antioxidant activity observed by the L/F extract in previous studies. Fucoidan may be a potential natural antioxidant to enhance lipid stability in meat products. The antioxidant potential of fucoidan and the L/F extract is strongly influenced by the cooking and digestion processes. The L/F extract demonstrated superior antioxidant activity compared to fucoidan in minced LTL, after cooking and post digestion. The antioxidant compounds of the L/F extract were partially absorbed by Caco-2 cells confirming their bioaccessibility post digestion. Results demonstrate the potential for extracts containing fucoidan to enhance antioxidant activity of functional cooked meat products as well as contribute to human antioxidant defence systems.

CHAPTER 8

General Discussion

The functional food market continues to grow in response to consumer demands for 'healthy' foods. Macroalgae contain a range of compounds with biological activity making them an ideal source of functional ingredients. Utilising the abundant supply of Irish macroalgae for functional food ingredients is currently the focus of a major research initiative in Ireland. Additionally, muscle foods are a potential carrier for functional ingredients, which would serve to improve and enhance the 'healthy' image of meat benefitting both producers and consumers alike. The objective of this thesis was to incorporate Irish macroalgae / macroalgal extracts rich in bioactive compounds, into muscle-based foods to investigate their potential as functional ingredients and subsequently develop functional meat products. The two main methods of incorporating ingredients with functionality into muscle foods included supplementation of fish/pig diets pre-slaughter and direct addition to pork meat during processing.

Initial studies examined whole macroalgae (*Ulva rigida* and *Palmaria palmata*) supplemented into fish diets. Commercially prepared macroalgal polysaccharide extracts (containing laminarin and fucoidan (wet- and spray-dried forms)) were added into pig diets at a number of levels and durations of feeding. In order to evaluate the efficacy of mode of incorporation (dietary versus direct addition), the spray-dried macroalgal extract was also added directly to minced pork meat. In addition to potential benefits on pork quality and shelf-life parameters, the functionality of polysaccharide extracts in cooked pork was further examined in terms of bioaccessibility, cellular uptake and transport using an *in vitro* digestion procedure and a Caco-2 cell (transwell) model. Mechanistic studies were also used to examine the antioxidant and pro-oxidant activities of laminarin and fucoidan alone and in combination.

In Chapters 2 and 3, two macroalgal species harvested from the shores of Ireland were selected: *Ulva rigida* (green) and *Palmaria palmata* (red) and included in Atlantic

salmon diets. The inclusion of macroalgae in fish diets to enhance fish growth and health parameters has been established for a variety of fish species; however the effect on fillet quality is not well investigated. Atlantic salmon diets are traditionally supplemented with synthetic carotenoids ((astaxanthin (red) and canthaxanthin (orange)) to produce the typical red/pink colour consumer's associate with fresh salmon. Both macroalgal species were selected as they are rich in carotenoids which may be a potential source of natural pigments for salmon fillets. Additionally, *Palmaria palmata* was selected due to the phycobiliproteins it contains which impart the characteristic red colour to the algae. Atlantic salmon fillets stored in modified atmosphere packs (60% N_2 : 40% CO_2) (MAP) for up to 15 days at 4ºC were analysed (proximate composition, pH, colour, lipid oxidation, microbiological analysis and sensorial analysis) to determine the effect of dietary macroalgae on fillet quality and shelf-life parameters.

Several factors influence the ability of salmon to deposit carotenoids in the muscle, including quantity available and carotenoid structure. In both dietary trials (Chapters 2 and 3), the salmon fillets were enhanced with a yellow/orange colour due to the deposition of carotenoids. Carotenoids (lutein and zeaxanthin) are essential for human eye health and the presence of carotenoids in salmon fillets could be promoted/marketed as a functional fish product. Unexpectedly, Atlantic salmon fed *Ulva rigida* resulted in a more intense yellow/orange fillet colour than salmon fed *Palmaria palmata*. This was likely due to the fact that water soluble phycobiliproteins (red) from *Palmaria palmata* are less likely to be deposited in fish muscle tissue compared to lipid soluble pigments. In addition, Atlantic salmon are poor depositors of pigments, from synthetic or natural sources, in comparison to other fish species and lack the ability to biotransform carotenoids into other structural forms which may confer appropriate coloration to salmon fillets. Other quality parameters examined were not negatively

influenced by dietary supplementation, indicating that macroalgae can be used as a natural ingredient source in Atlantic salmon feed. Furthermore, macroalgae may be used in Atlantic salmon diets to reduce the use of artificial orange colour sources (i.e. canthaxanthin) currently used in aquaculture practices. The use of more purified or refined macroalgal extracts in the salmon feed to increase deposition of biologically active compounds in salmon fillets may be required to further enhance other quality parameters such as lipid stability.

Commercial macroalgal extracts contain one or more compounds/components present in seaweed, in a concentrated form. Macroalgal polysaccharide extracts containing laminarin and fucoidan, from *Laminaria digitata*, were recently shown to possess benefits in terms of porcine gut health (O'Doherty et al., 2010). The first trial (Chapter 4) examined two forms: wet- and spray-dried macroalgal polysaccharide extracts (containing laminarin and fucoidan) (laminarin, 500 mg/kg feed; fucoidan, 420 mg/kg feed). Porcine diets were supplemented for 21 days pre-slaughter. The effects of dietary supplementation on quality and shelf-life parameters (pH, colour, lipid oxidation, microbiological analysis) of fresh *longissimus thoracis et lumborum* (LTL) steaks stored in MAP (80% O_2 : 20% CO_2) for up to 15 days at 4ºC was examined.

Although results were not statistically significant ($p > 0.05$), decreased levels of lipid oxidation in the LTL muscle were observed indicating deposition of antioxidant components from the wet- and spray-dried extracts in pork muscle. The wet-extract was more effective than the spray-dried macroalgal extract possibly due to the impact of the temperature used during industrial spray drying of the wet extract. Liver, heart, kidney and lung tissue homogenates were subjected to iron ($FeSO_4$)-induced lipid oxidation to determine if antioxidant compounds from the macroalgal extract were distributed elsewhere in the animal. Although not significant, lower levels of lipid oxidation were

observed in the liver tissue homogenates indicating deposition of antioxidant compounds in specific organ tissues.

Level (450 or 900 mg laminarin and fucoidan/kg feed) and duration (3 or 6 weeks) of feeding the wet macroalgal polysaccharide (L/F-WS) extract on the quality and shelf-life of fresh pork was subsequently assessed in Chapter 5. Supplementation of both levels of the L/F-WS extract for 3 weeks was more effective than 6 weeks at decreasing lipid oxidation in fresh pork steaks. Over time, porcine gut microbiota can become accustomed to macroalgal polysaccharides. This could result in more breakdown and utilisation of the macroalgal extract by gut microbiota prior to transportation to the muscle. Furthermore, trends indicated that levels of lipid oxidation in cooked pork patties were lower, although results were not statistically significant. The behaviour of dietary seaweed polysaccharides during cooking requires further investigation as confirmation of antioxidant activity post-cooking would be required for bioactivity.

The mechanism of action of the L/F-WS extract on lipid stability was also explored. Trends indicated that the dietary L/F-WS extract lowered the levels of saturated (∑SFA) fatty acids in pork meat. Pork meat products with improved fatty acid profiles and enhanced lipid stability may benefit consumers seeking meat with reduced saturated fat levels. The antioxidant activity of the L/F-WS extract in pork muscle was attributed to a combination of proposed mechanisms including: immunomodulatory activity in the porcine gut, influence on the fatty acid composition (lower levels of saturated fatty acids) and free radical scavenging activity.

In order to compare efficacy of mode of incorporation (dietary supplementation versus direct addition) the spray-dried macroalgal polysaccharide (L/F-SD) extract containing laminarin and fucoidan was added directly to pork meat and the effects on quality and shelf-life parameters on pork patties were examined in Chapter 6. In contrast

to the dietary supplementation studies, the L/F-SD extract decreased surface redness and increased lipid oxidation in fresh pork patties. An interaction between pork meat constituents (myoglobin) and the L/F-SD extract was purposed as the contributing factor in decreased surface redness of the pork patties. The lipid pro-oxidant activity of the L/F-SD extract was attributed to the other compounds present in the extract such as iron, copper and salt. In contrast to the lipid pro-oxidant activity observed in fresh pork patties, levels of lipid oxidation decreased in cooked pork patties due to the addition of the L/F-SD extract. This was attributed to the formation of Maillard reaction products not present in the fresh pork meat (containing L/F-SD) and indicated heating could enhance the bioactivity of the L/F-SD extract in cooked muscle foods.

The ability of macroalgal polysaccharide extracts containing laminarin and fucoidan to enhance cooked pork meat quality but promote lipid oxidation in fresh pork patties (when added directly) was determined in Chapters 5 and 6. Therefore an initial investigation in Chapter 7 focussed on exploring the anti-oxidative potential of purified laminarin and fucoidan individually and in comparison to the L/F-SD extract utilised in Chapters 4 and 6. Separately, fucoidan demonstrated superior antioxidant activity to laminarin in pork meat homogenates; however fucoidan promoted oxymyoglobin oxidation in solution (commercial horse heart oxymyoglobin). The mechanism by which fucoidan decreased lipid oxidation but promoted oxymyoglobin oxidation was attributed to the presence of sulphate groups which can scavenge free radicals as well as oxidise ferrous iron to the ferric state. The interaction between fucoidan and oxymyoglobin may have contributed to the decreased surface redness of pork patties containing the L/F-SD extract observed previously in Chapter 6.

Results from Chapters 5 and 6 indicated moderate thermal stability and enhanced antioxidant activity of the L/F-SD extract in cooked pork meat. Therefore in Chapter 7,

the final investigation of this thesis was to determine the bioaccessibility (fraction available for absorption and uptake) of antioxidant compounds from the L/F-SD extract in pork patties following cooking and post digestion. Cooking negatively affected the DPPH free radical scavenging activity of fucoidan in cooked pork patties. Conversely, the DPPH free radical scavenging activity of the L/F-SD extract was enhanced due to the formation of Maillard reaction products in the cooked pork patties. *In vitro* digestion of the cooked pork patties containing the L/F-SD extract increased DPPH free radical scavenging activity. The increased DPPH free radical scavenging activity of the L/F-SD extract was attributed partially to fucoidan as well as potential synergy between other components in the extract such as protein and mannitol. Following cellular uptake, DPPH free radical scavenging activity decreased following incubation with Caco-2 cells indicating uptake and transport of antioxidant compounds. Functional cooked meat products formulated with the L/F-SD extract could contribute to the antioxidant defence system in humans and prove beneficial to human health.

In summary, the inclusion of L/F-WS and L/F-SD extracts in porcine diets exhibited quality enhancing effects in fresh and cooked pork steaks. The addition of the L/F-SD extract enhanced lipid stability in cooked pork patties. The mode of incorporation by which macroalgal polysaccharide extracts are added to meat appears to influence the mode of action of the bioactive components present. The pro-oxidant effects of the L/F-SD extract in fresh pork may be eliminated due to porcine digestive and metabolic processes. Fucoidan and the L/F-SD extract demonstrated bioactivity (*in vitro* antioxidant assays). Bioaccessibility of antioxidant activity of the L/F-SD extract after cooking and post digestion was confirmed using an *in vitro* digestion and cellular uptake model.

Overall Conclusions

- Results indicated the potential use of macroalgae and commercial macroalgal polysaccharide extracts as potential functional ingredients in muscle foods.
- *Ulva rigida* and *Palmaria palmata* may be considered as replacement functional ingredients for Atlantic salmon feed without negatively impacting fillet quality and shelf-life parameters.
- Deposition of carotenoids (lutein and zeaxanthin) with bioactivity in Atlantic salmon fillets could reduce the use of synthetic pigments such as canthaxanthin, and salmon could subsequently be marketed as a functional fish product.
- The inclusion of macroalgal polysaccharide extracts containing laminarin and fucoidan into animal feed could enhance pork meat quality.
- Laminarin and fucoidan may be used as a natural antioxidant alternative in the development of novel functional meat products.
- The use of macroalgal polysaccharide extracts containing laminarin and fucoidan added directly to fresh meat could enhance cooked pork meat quality.
- Antioxidant activity of the macroalgal polysaccharides was enhanced by the *in vitro* digestion process.
- Bioaccessibility of antioxidant compounds of the spray-dried macroalgal extract confirmed the use of macroalgal polysaccharides as functional ingredients in muscle based foods.

Future Research

This thesis provides preliminary investigations into the use and effects of macroalgae and macroalgal polysaccharides in muscle foods. The use of macroalgae as natural ingredients in food has the potential to improve quality and enhance food safety. Research on macroalgae and macroalgal extracts in food is limited with abundant opportunity to further our knowledge of this valuable resource. Future research needs arising from the work presented in this thesis are summarised as follows:

- Further elucidation of the specific structure and antioxidant mechanisms of laminarin and fucoidan to provide a better understanding of their behaviour and functionality in meat products.
- Examine the effect of fucoidan and the spray-dried macroalgal polysaccharide extract in other fresh and cooked meat products.
- Investigate the use of other macroalgal compounds with antioxidant activity (i.e. fucoxanthin) in muscle foods.
- Confirm bioaccessibility and bioavailability of other macroalgal derived functional ingredients in muscle based foods after cooking and post digestive processes.
- Conduct *in vivo* human dietary trials in order to validate *in vitro* studies.
- Increase consumer awareness about the health benefits of macroalgae and sensory changes associated with incorporation of macroalgae into muscle products.
- Develop cost effective harvesting and extraction processes necessary to facilitate the use of macroalgae as ingredients in muscle foods.

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Appendix

Publications

Research Article

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Inclusion of *Palmaria palmata* (red seaweed) in Atlantic salmon diets: effects on the quality, shelf-life parameters and sensory properties of fresh and cooked salmon fillets

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Abstract

BACKGROUND: The use of *Palmaria palmata* (PP) as a natural ingredient in farmed Atlantic salmon diets was investigated. The effect of salmon diet supplementation with *P. palmata* (0, 5, 10 and 15%) or synthetic astaxanthin (positive control, PC) for 16 weeks pre-slaughter on quality indices of fresh salmon fillets was examined. The susceptibility of salmon fillets/homogenates to oxidative stress conditions was also measured.

RESULTS: In salmon fillets stored in modified atmosphere packs (60% N_2/40% CO_2) for up to 15 days at 4 °C, *P. palmata* increased surface −*a (greenness) and *b** (yellowness) values in a dose-dependent manner, resulting in a final yellow/orange flesh colour. In general, the dietary addition of *P. palmata* had no effect on pH, lipid oxidation (fresh, cooked and fillet homogenates) and microbiological status. 'Eating quality' sensory descriptors (texture, odour and oxidation flavour) in cooked salmon fillets were not influenced by dietary *P. palmata*. Salmon fed 5% PP showed increased overall acceptability compared with those fed PC and 0% PP.**

CONCLUSION: Dietary *P. palmata* was ineffective at providing red coloration in salmon fillets, but pigment deposition enhanced fillets with a yellow/orange colour. Carotenoids from *P. palmata* may prove to be a natural pigment alternative to canthaxanthin in salmon feeds.

Keywords: seaweed; *Palmaria palmata*; pigments; farmed Atlantic salmon; feed ingredient; fillet quality

INTRODUCTION

The current worldwide production of farmed Atlantic salmon (*Salmo salar*) has increased substantially to over 1×10^6 t with the increased demand for highly nutritious fresh salmon and salmon-related products in Japan, the European Union and North America.[1] Wild salmon feed on other fish and small crustaceans such as krill containing carotenoid pigments, which are responsible for the red/pink salmon colour consumers associate with acceptable salmon fillet quality.[2] Since salmon cannot synthesise carotenoids, farmed salmon diets are mostly supplemented with synthetic sources of carotenoids (mainly astaxanthin and to a lesser extent canthaxanthin) in order to pigment farmed salmon flesh similar to wild salmon.[3]

In nature, astaxanthin (red) exists as three optical isomers (3*R*,3′*R*, 3*S*,3′*S* and 3*R*,3′*S*), with varying ratios depending on the source. The 3*R*,3′*R* isomer is considered most bioavailable, and all three isomers are present in different ratios in wild salmon. Synthetic astaxanthin (usually an isomer ratio of 1:2:1) is absorbed, transported and deposited in salmon flesh similarly to natural forms of astaxanthin.[4] Canthaxanthin (orange) is not found in wild Atlantic salmon but is a minor carotenoid in Pacific salmon species and is used in some farmed salmon feeds in conjunction with astaxanthin.[5]

The colour of salmon is regarded as one of the most important quality criteria after freshness.[5] Pigmentation from dietary carotenoids (yellow, orange and red) can range from pale, faintly pigmented flesh to strong red tones and is considered a vital aspect of commercial feed formulation and fish management. In addition to flesh pigmentation, carotenoids have been linked to proper growth and survival as well as to possible protection of tissues from oxidative damage.[6] Synthetic pigments traditionally

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used in salmon feed continue to be utilised with success, but pigments from natural sources should be considered with the increase in consumer preference for natural food additives.[7]

Historically, farmed salmon were fed a diet composed of fish meal (sole protein source) and fish oil (major dietary lipid source). In order to increase sustainability and decrease production costs in aquaculture, substitution of fish meal with alternative protein sources such as soybean, corn gluten and wheat has been investigated previously. Today, many alternative plant protein sources are successfully incorporated into fish feeds.[8,9] However, to date, limited research has been conducted examining the nutritional value of seaweeds (macroalgae) as a potential protein substitute for fish meal replacement. Several sources of biological astaxanthin have been utilised to address consumer demand for natural pigmentation of fish, including yeast (*Phaffia rhodozyma*), krill (*Euphausia superba*) or crab wastes and green microalgae (*Haematococcus pluvialis*).[10]

The average protein content of the red seaweed (Rhodophyta) *Palmaria palmata* (~35%) is comparable to that of high-protein vegetables such as soybeans and, as such, may be used as an alternative protein source for fish feeds.[11] Ten amino acids essential for salmon growth and protein structure are all present in *P. palmata*.[12,13] *Palmaria palmata* also contains a variety of fat-soluble carotenoids, including high levels of lutein (yellow), α- and β-carotene (reddish yellow) as well as chlorophyll *a*/*b* (mid green to olive green).[14,15] Carotenoids are isoprenoid molecules that aid in the absorption of sunlight and protect cells from oxidative stress by quenching singlet oxygen.[4] The carotenoid content of *P. palmata* varies seasonally, depends on postharvest treatment and is generally higher than that in other seaweed species.[13] Red seaweeds differ in structure and photosynthetic pigments from green and brown seaweeds, with the red colour attributed to the presence of water-soluble light-harvesting pigments known as phycobiliproteins, found in chloroplasts, which mask the other fat-soluble pigments. There are three major classes of phycobiliproteins: phycoerythrin (red), phycocyanin (blue) and allophycocyanin (green/blue).[16]

Individually, colour pigments (carotenoids, xanthophylls, chlorophyll and phycobiliproteins) found in *P. palmata* demonstrated potent antioxidant activity in a number of test systems, including *in vitro* antioxidant assays (DPPH, ABTS) and the inhibition of conjugated dienes and TBARS products in a linoleic acid emulsion.[17,18] Pigments have also been reported to possess a range of health-promoting properties. Carotenoids and chlorophyll derivatives have exhibited anticancer properties, while phycobiliproteins have been linked to significant anti-inflammatory, hepatoprotective and free radical-scavenging properties.[17,19–21]

Seaweeds may offer potential for use as a fish meal replacer (protein source) and a source of natural pigments and bioactive compounds in fish diets. Therefore the use of seaweed in salmon diets may satisfy the increased consumer demand for natural, health-promoting products and merits investigation. The objective of this study was to examine the effect of including *P. palmata* (0–15%) in farmed salmon diets on the quality, shelf-life parameters and sensory properties of salmon fillets.

MATERIALS AND METHODS

Reagents

All chemicals used were of 'AnalaR' grade and were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), Oxoid Ltd (Basingstoke, UK) or Merck KGaA (Darmstadt, Germany). *Palmaria palmata* was harvested from the coasts of Galway Bay, Ireland.

Salmon and diets

After harvesting, *P. palmata* was washed and dried before addition to the salmon diet formulations. Diets were prepared in the feed formulation laboratory at Carna Research Station, Ryan Institute, National University of Ireland, Galway. All diets were formulated to be iso-nitrogenous (40%), iso-lipidic (25%) and iso-caloric (26 MJ kg^{-1}). The composition of the experimental diets is outlined in Table 1.

Atlantic salmon smolts (*S. salar*) were obtained from a commercial company based at Lough Fee, Connemara, Ireland. After an 8 week acclimatisation period, a 16 week completely randomised experimental feed trial was carried out at Carna Research Station. Salmon ($n = 33$, average fish weight ~170.4 g) were randomly assigned to one of 15 tanks (three tanks per treatment) that consisted of five different experimental formulated diets. The positive

Table 1. Composition (g kg^{-1}, unless indicated otherwise) of experimental salmon diets with inclusion of *Palmaria palmata* (PP) (adapted from Wan *et al.*[22])

Component	PC	0% PP	5% PP	10% PP	15% PP
Fish meal[a]	407.4	407.4	390.8	374.1	357.5
Fish oil[a]	200.0	200.0	201.4	202.8	204.1
Palmaria palmata	–	–	50.0	100.0	150.0
Corn starch[b]	187.3	187.6	152.8	118.1	83.4
Lysamine[c]	90.0	90.0	90.0	90.0	90.0
Glutalys[c]	90.0	90.0	90.0	90.0	90.0
Mineral and vitamin premix[d]	20.0	20.0	20.0	20.0	20.0
Lucantin Pink[e]	0.3	–	–	–	–
Barox Plus[f]	5.0	5.0	5.0	5.0	5.0

[a] United Fish Products Ltd, Donegal, Ireland.
[b] Laboratory grade, Sigma-Aldrich Company Ltd, Poole, UK.
[c] Purified feed ingredients, Roquette, Lestrem, France.
[d] Premier Nutrition Products Ltd, Rugeley, UK (manufacturer's analysis: Ca, 12.09%; ash, 78.708%; Na, 8.858%; vitamin A, 1.0 µg kg^{-1}; vitamin D3, 0.10%; vitamin E, 7.0 g kg^{-1}; Cu, 250 mg kg^{-1}; Mg, 15.6 g kg^{-1}; P, 5.2 g kg^{-1}).
[e] BASF, Ludwigshafen, Germany.
[f] Kemin Europa NV, Herentals, Belgium.

control group (PC) was fed a basal diet plus synthetic astaxanthin (0.3 g kg^{-1} feed) (Table 1). The remaining four groups were fed the basal diet plus *P. palmata* (PP) at 0, 5, 10 and 15% PP inclusion levels. The salmon were hand-fed on five occasions over the course of each day and housed in 1000 L tanks fed by a filtered flow-through seawater supply (ambient temperature regime). At the end of the feeding trial, salmon (average fish weight ~419.9 g) were euthanised with a sharp blow to the head followed by pithing of the brain and were gutted. Fresh (raw) salmon fillets were transported on ice at 4 °C to the School of Food and Nutritional Sciences, University College Cork, Ireland. Further details of the experimental site, feeding trial conditions and growth of fish are reported in Wan *et al.*[22]

Proximate analysis of fresh salmon fillets

The proximate composition of fresh salmon fillets was reported on a wet weight basis. Salmon fillet protein content (N × 6.25) was determined by the Kjeldahl method.[23] Moisture and fat contents were measured using a SMART Trac rapid moisture/fat analyser (CEM Corporation, Matthews, NC, USA). Ash content was determined using a muffle furnace.[24] Compositional analysis results were expressed as g per 100 g wet weight.

Salmon processing and packaging

Fresh salmon fillets (~100 g) (PC, 0, 5, 10 and 15% PP) were placed in low-oxygen-permeable (<1 $cm^3\ m^{-2}\ day^{-1}$ at STP) polystyrene/ethylvinylalcohol/polyethylene trays and, employing modified atmosphere packaging technology, flushed with 60% N_2/40% CO_2 using a vacuum-sealing unit (VS 100, Gustav Müller and Co. KG, Bad Homburg, Germany) equipped with a gas mixer (Witt-Gasetechnik GmbH and Co. KG, Witten, Germany). Trays were covered and heat-sealed using a low-oxygen-permeable (3 $cm^3\ m^{-2}\ day^{-1}$ at STP) laminated barrier film with a polyolefin heat-sealable layer. Fresh salmon fillets were stored for up to 15 days under fluorescent lighting conditions (~660 lx) at 4 °C. The gas atmosphere (% O_2 and % CO_2) in the modified atmosphere packs (MAPs) was monitored using a CheckMate 9900 (PBI-Dansensor, Ringsted, Denmark). Immediately after gas flushing, MAPs contained 56.91 ± 0.53% N_2 and 42.95 ± 0.42% CO_2. The average gas composition in MAPs at the end of the 15 day storage period was 60.40 ± 1.66% N_2/39.60 ± 1.66% CO_2; % N_2 was calculated by difference of % O_2 and % CO_2.

In a cooked fish study, salmon fillets from all treatments (PC, 0, 5, 10 and 15% PP) were placed on aluminium foil-lined trays and cooked at 180 °C for 12 min in a fan-assisted convection oven (Zanussi Professional 10 GN1/1, Conegliano, Italy) until an internal temperature of 72 °C was reached. Cooked fillets were placed in trays over-wrapped with oxygen-permeable film and stored aerobically for up to 5 days at 4 °C.

Measurement of pH

Fresh salmon fillet samples (10 g) were homogenised for 1 min at 24 000 rpm in 90 mL of distilled water using an Ultra Turrax T25 homogeniser (Janke and Kunkel, IKA-Labortechnik GmbH and Co., Staufen, Germany) and the pH was measured at 20 °C using a pH meter (Seven Easy portable, Mettler-Toledo GmbH, Schweizenbach, Switzerland). The pH of salmon fillets was recorded on days 1, 3, 7, 12 and 15 of storage.

Colour measurement

Surface colour was measured using a Konica Minolta CR-400 Chroma Meter (Minolta Camera Co., Osaka, 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 on the CIE LAB colour space system using a white tile (D_c: $L = 97.79$, $a = -0.11$, $b = 2.69$). The L^* value represents lightness and a^* and b^* values represent redness and yellowness respectively. Colour measurements of fresh salmon fillets were recorded on days 1, 3, 7, 12 and 15.

Measurement of lipid oxidation

Lipid oxidation was measured using the 2-thiobarbituric acid (TBA) assay of Siu and Draper.[25] The malondialdehyde (MDA) content was calculated using an extinction coefficient of $1.56 \times 10^5\ L\ mol^{-1}\ cm^{-1}$. Results were expressed as 2-thiobarbituric acid-reactive substances (TBARS) in mg MDA kg^{-1} salmon. Lipid oxidation was measured in fresh salmon fillets on days 1, 3, 7, 12 and 15 of storage and in cooked salmon fillets on days 0, 1, 3 and 5.

Preparation of salmon fillet homogenates

Fresh salmon fillet homogenates (25%) were prepared by homogenising 25 g of tissue in 75 mL of 0.12 mol L^{-1} KCl/5 mmol L^{-1} histidine (pH 5.5) using an Ultra Turrax T25 homogeniser at 24 000 rpm for 3 min. Lipid oxidation in homogenate incubates (30 mL) was initiated with equimolar ferric chloride ($FeCl_3$)/sodium ascorbate (45 µmol L^{-1}). Homogenates without $FeCl_3$ and ascorbic acid were run simultaneously as controls. Lipid oxidation in fresh salmon homogenates was measured immediately (time 0) and after 1, 4 and 24 h of storage at 4 °C.

Measurement of lipid oxidation in salmon fillet homogenates

A modification of the TBA assay of Siu and Draper[25] was used to measure lipid oxidation in fresh salmon fillet homogenates. Homogenate samples (4 mL) were added to 4 mL of 100 g L^{-1} trichloroacetic acid. Samples were mixed using a vortex mixer and the precipitate formed was removed by filtering through Whatman No. 1 filter paper. In a screw-cap test tube, 4 mL of clear supernatant was added to 1 mL of 0.06 mol L^{-1} TBA. The tube was incubated at 80 °C for 90 min and the absorbance of the resulting coloured complex was measured using a spectrophotometer at 532 nm against a blank containing all reagents and distilled water instead of the filtrate. The MDA content was calculated using an extinction coefficient of $1.56 \times 10^5\ L\ mol^{-1}\ cm^{-1}$. Results were expressed as TBARS in mg MDA kg^{-1} salmon.

Microbiological analysis

Fresh salmon fillet samples (10 g) were transferred into stomacher bags, diluted with 90 mL of maximum recovery diluent and stomached (Steward Stomacher 400 Lab Blender, London, UK) for 3 min, resulting in a 10^{-1} dilution used for analysis. Serial dilutions were prepared and 0.1 mL aliquots from each dilution were plated onto standard plate count agar (Oxoid Ltd). Plates were incubated at 30 °C for 48 h and at 4 °C for 10 days to determine mesophilic and psychrotrophic counts respectively. Microbiological analysis of fresh salmon fillets was carried out on days 1, 3, 7, 12 and 15 of storage. Results were expressed as $\log_{10}$ colony-forming units (CFU) g^{-1} salmon.

Table 2. Effect of dietary *Palmaria palmata* (PP) on proximate composition of fresh salmon fillets ($n = 3$)

Treatment	Proximate composition (g per 100 g wet weight)			
	Protein	Moisture	Fat	Ash
PC	21.13 ± 0.82[a]	75.45 ± 1.56[a]	2.13 ± 0.89[a]	1.29 ± 0.12[a]
0% PP	21.01 ± 0.61	75.49 ± 1.40	2.27 ± 1.02	1.23 ± 0.19
5% PP	22.00 ± 0.59	73.74 ± 1.90	2.85 ± 1.45	1.40 ± 0.22
10% PP	22.45 ± 1.26	74.40 ± 1.77	1.83 ± 0.74	1.32 ± 0.12
15% PP	20.52 ± 0.66	74.89 ± 2.14	3.32 ± 1.71	1.27 ± 0.12

[a] Within each composition type, mean values (±standard deviation) in the same column are not significantly different ($P > 0.05$).

Sensory evaluation

Sensory analysis ('visual' and 'eating quality') of fresh and cooked salmon fillets stored in 60% N_2/40% CO_2 was performed in duplicate by 26 naïve assessors on days 1 and 7 of storage following the method of O'Sullivan *et al.*[26] By day 12, the bacterial count was deemed unsafe to continue further sensory evaluation. 'Visual' sensory analysis descriptors examined were pinkness, whiteness, drip, purchasing appeal and overall acceptability. 'Eating quality' sensory analysis descriptors examined were colour, texture, odour, oxidation flavour 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).

Salmon fillets were cooked for sensory analysis in a Zanussi oven at 180 °C for 12 min until an internal meat temperature of 72 °C was reached. Following cooking, fillets were cooled, the fish was removed from the skin and salmon portions were identified with random three-digit codes. Sample presentation order was randomised to prevent any flavour carryover effects.[27] Sensory analysis was undertaken in the panel booths at the university sensory laboratory in accordance with ISO regulations.[28] Assessors were also provided with water and crackers to cleanse their palates between samples. Results for sensory analysis scores were measured in cm. Results were presented as significance of regression coefficients.

Statistical analysis

All analyses were performed in duplicate. Mean sample values ($n = 3$) for each of the five treatment groups (PC, 0, 5, 10 and 15% PP) were subjected to statistical analysis. A full repeated measures two-way analysis of variance (ANOVA) was conducted to investigate the effects of dietary *P. palmata* level, time and their interaction. Dietary *P. palmata* level represented the 'between-subjects' factor, while the effect of time was measured using the 'within-subjects' factor. Tukey's test was used to adjust for multiple comparisons between treatment means. The analysis was carried out using SPSS 18.0 for Windows (SPSS, Chicago, IL, USA).

'Visual' and 'eating quality' sensory data were analysed with ANOVA/partial least squares regression (APLSR) to process the mean data accumulated from the 26 test subjects in duplicate. 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 APLSR models presented was determined to be six principal components. In these models, assessor- and session-level effects were removed using level correction. The validated explained variance for the model constructed was −0.51 and 6.27% and the calibrated variance was 2.38 and 8.97% on days 1 and 7 respectively. To derive significance indications for the relationships determined in the quantitative APLSR, regression coefficients were analysed by jack-knifing based on cross-validation and stability plots.[29,30] All analyses were performed using The Unscrambler Version 9.8 (CAMO ASA, Trondheim, Norway).

RESULTS AND DISCUSSION

Proximate analysis and pH of salmon fillets

The protein contents of fresh (raw) salmon fillets from fish fed iso-nitrogenous diets ranged from ∼20 to 22 g per 100 g for all dietary treatments (Table 2). Similarly, Bjerkeng *et al.*[9] reported a protein content of ∼18 g per 100 g for Atlantic salmon fillets from fish fed fish meal or soybean as a fish meal replacer. Levels of fat ranged from ∼2 to 3 g per 100 g, with no significant differences between dietary treatments. In a similar study, the lipid content of *Oncorhynchus mykiss* (rainbow trout) fillets was not affected by dietary addition of *Porphyra dioica* (red alga) at levels ranging from 0 to 15%.[31] Moisture and ash levels were unaffected by *P. palmata* supplementation, with levels ranging from ∼74 to 76 g per 100 g and from ∼1.2 to 1.4 g per 100 g respectively. Several studies have reported no effect on salmon fillet proximate composition when plant-based meals are used as alternative protein and lipid sources compared with traditional feed (fish meal and oil).[9,32] In the present study, the proximate analysis of salmon fillets from salmon fed *P. palmata* is comparable to that from salmon fed the positive control (PC) diet. Therefore *P. palmata* may be used as a potential replacement functional ingredient for fish meal in Atlantic salmon feed without detrimentally affecting the proximate composition of salmon fillets.

The pH of fresh salmon fillets ranged from ∼6.27 to 6.33 over the 15 day storage period and was unaffected by the addition of dietary *P. palmata*. Similarly, Einen *et al.*[33] reported fillet pH stability (6.3) during storage of Atlantic salmon (*S. salar*) for up to 100 h.

Colour stability of fresh salmon fillets

Trends indicated that the surface lightness (L^*) values of fresh salmon fillets increased over the storage time (Table 3). Lightness values of the PC group were significantly lower ($P < 0.05$) compared with 15% PP on day 1 and with 0 and 10% PP on day 7 of storage. The lower L^* values of PC fillets were attributed to the concurrent increase in flesh pigment concentration provided by deposition of synthetic astaxanthin in the fish muscle. Buttle *et al.*[34] reported a similar pattern of decreased lightness in fresh fillets from Atlantic salmon fed astaxanthin, canthaxanthin or an astaxanthin/canthaxanthin mix (0.06 g kg^{-1} feed).

Table 3. Effect of dietary *Palmaria palmata* (PP) on surface lightness (L^*), redness ($+a^*$), greenness ($-a^*$) and yellowness (b^*) values of fresh salmon fillets ($n = 3$) stored in modified atmosphere packs (60% N_2/40% CO_2) for up to 15 days at 4 °C

		Storage time at 4 °C (days)				
Treatment	Parameter	1	3	7	12	15
PC		41.29 ± 2.06^{a}	44.72 ± 1.81^{a}	45.09 ± 2.13^{b}	48.21 ± 1.76^{a}	48.75 ± 3.86^{a}
0% PP		44.73 ± 2.35^{a}	45.54 ± 2.05^{a}	50.42 ± 1.23^{a}	51.03 ± 2.05^{a}	49.67 ± 2.05^{a}
5% PP	L^*	46.78 ± 2.11^{a}	45.82 ± 0.50^{a}	49.78 ± 3.01^{a}	50.56 ± 0.90^{a}	49.88 ± 2.09^{a}
10% PP		46.85 ± 2.79^{a}	47.44 ± 3.30^{a}	50.49 ± 0.97^{a}	49.59 ± 1.36^{a}	50.02 ± 3.00^{a}
15% PP		48.24 ± 1.40^{b}	45.72 ± 1.00^{a}	49.28 ± 0.25^{a}	49.21 ± 0.62^{a}	52.10 ± 1.73^{a}
PC		5.63 ± 1.47^{b}	6.69 ± 1.61^{b}	6.38 ± 1.79^{b}	6.08 ± 0.84^{b}	8.29 ± 1.32^{b}
0% PP		-0.61 ± 0.41^{a}	-0.53 ± 0.43^{a}	-0.63 ± 0.27^{a}	-1.07 ± 0.15^{a}	-0.48 ± 1.02^{a}
5% PP	a^*	-1.21 ± 0.26^{a}	-1.07 ± 0.68^{a}	-0.55 ± 0.82^{a}	-1.11 ± 1.24^{a}	-1.01 ± 0.15^{a}
10% PP		-1.54 ± 0.28^{a}	-0.54 ± 1.01^{a}	-0.71 ± 0.40^{a}	-1.22 ± 0.64^{a}	-2.29 ± 0.09^{a}
15% PP		-1.35 ± 0.59^{a}	-1.49 ± 0.18^{a}	-1.27 ± 0.95^{a}	-1.49 ± 0.89^{a}	-2.11 ± 0.36^{a}
PC		10.84 ± 0.92^{b}	11.50 ± 2.00^{b}	11.10 ± 0.76^{b}	12.81 ± 0.73^{b}	12.81 ± 1.47^{b}
0% PP		2.52 ± 0.38^{a}	2.58 ± 0.47^{a}	4.16 ± 1.83^{a}	4.05 ± 0.65^{a}	4.98 ± 0.16^{a}
5% PP	b^*	3.88 ± 0.46^{a}	5.05 ± 0.31^{ac}	6.12 ± 1.57^{ac}	6.14 ± 1.43^{ac}	5.59 ± 0.37^{a}
10% PP		6.54 ± 0.41^{c}	7.47 ± 0.15^{cd}	8.60 ± 0.95^{bc}	8.00 ± 1.69^{c}	7.92 ± 1.73^{ac}
15% PP		8.98 ± 1.32^{b}	8.58 ± 0.58^{d}	10.43 ± 0.37^{b}	8.96 ± 1.54^{c}	11.01 ± 2.40^{bc}

[a,b,c,d]Within each parameter and storage day, mean values (±standard deviation) in the same column bearing different superscripts are significantly different.

In general, the surface redness (a^*) values of PC salmon fillets increased as a function of storage time (Table 3). The increased surface redness due to synthetic astaxanthin deposited in the PC salmon fillets resulted in a red/pink colour. In the fillets from salmon fed *P. palmata* (0–15% PP), the surface greenness ($-a^*$) increased as a function of storage time and *P. palmata* concentration. The increase in surface greenness as a function of dietary *P. palmata* indicated deposition of pigments that were ineffective at providing red coloration. Similarly, Chatzifotis *et al.*[35] reported the inefficacy of the red carotenoid lycopene, contained in tomatoes, in providing red coloration in red porgy (*Pagrus pagrus*). *Palmaria palmata* contains other pigments available for absorption and deposition in the fish muscle, such as chlorophyll *a*/*b* (green) and phycobiliproteins, including *R*-phycocyanin (greenish blue), allophycocyanin (greenish blue), *R*-phycoerythrin (red) and *β*-phycoerythrin (red).[36] In the present study, chlorophyll, *R*-phycocyanin and allophycocyanin, all of which exhibit green colour, may have contributed to the final greenness colour observed in the salmon fillets.

General trends showed that the surface yellowness (b^*) values increased over the storage time as a function of *P. palmata* level on all storage days. Yellowness values were significantly ($P < 0.05$) higher for 10 and 15% PP than for 0% PP on each storage day (Table 3). On days 1, 7 and 15, the b^* values of PC and 15% PP were similar. In a previously reported study, rainbow trout fed diets containing *P. dioica* (0–15%) with no added astaxanthin exhibited stronger orange flesh tones in fillets as a function of the red alga concentration; it was concluded that the red alga contained fat-soluble yellow xanthophylls, especially lutein and zeaxanthin, which contributed to the final flesh coloration.[31] Olsen and Baker[37] reported the absorption and deposition of lutein, structurally similar to astaxanthin, in Atlantic salmon muscle. In the present study, the increased surface yellowness is most likely due to deposition of fat-soluble carotenoids, mainly lutein, from *P. palmata*.

Several factors influence carotenoid deposition in the fish muscle, including quantity available, carotenoid structure and the ability of salmon to metabolise or biotransform available carotenoids. The final carotenoid structure and level of deposition will determine the final colour of the fillet.[10] In the present study, the carotenoid profile (lutein, *α*- and *β*-carotene) exclusive to *P. palmata* was not sufficient to increase surface redness of the salmon fillets, but the carotenoids did enhance surface yellowness. In recent years, fish feed ingredients have included many plant sources such as maize gluten with significant amounts of yellow pigments from carotenoids such as lutein and zeaxanthin.[38] Similarly, canthaxanthin, sometimes used in farmed salmon feed, imparts an orange colour to salmon flesh and is therefore used in conjunction with astaxanthin to achieve desired final red/pink pigmentation.[34] Owing to the final yellow/orange flesh colour of salmon fillets in the present study, the carotenoids from *P. palmata* may be considered as a potential natural pigment alternative to the use of canthaxanthin in farmed salmon feed.

Lipid oxidation in fresh and cooked salmon fillets and fresh salmon fillet homogenates

Overall, the levels of lipid oxidation in fresh salmon fillets stored in MAPs were low, with mean values ranging from 0.07 to 0.11 mg MDA kg^{-1} salmon (Table 4). These low levels of lipid oxidation may be attributed to the physical condition of the salmon fillets (intact) and the gaseous environment within the MAPs (60% N_2/40% CO_2). Randell *et al.*[39] also reported low lipid oxidation values (<1 mg MDA kg^{-1}) for salmon fillets stored in MAPs (60% CO_2/40% N_2 or 40% CO_2/ 60% N_2) at 2 °C. Processes that change the physical structure of muscle foods, e.g. grinding or mincing and cooking, accelerate lipid oxidation reactions. In cooked salmon fillets, lipid oxidation increased over the 5 day storage period (Fig. 1) and levels of lipid oxidation were higher than in fresh salmon fillets, with mean values ranging from 0.33 to 1.29 mg MDA kg^{-1} salmon. The addition of *P. palmata* to salmon diets did not significantly influence lipid oxidation in fresh or cooked salmon fillets.

Table 4. Effect of dietary *Palmaria palmata* (PP) on lipid oxidation (TBARS, mg MDA kg^{-1} salmon) in fresh salmon fillets ($n = 3$) stored in modified atmosphere packs (60% N_2/40% CO_2) for up to 15 days at 4 °C

	Storage time at 4 °C (days)				
Treatment	1	3	7	12	15
PC	0.103 ± 0.02[a]	0.073 ± 0.01[a]	0.098 ± 0.00[a]	0.071 ± 0.01[a]	0.101 ± 0.01[a]
0% PP	0.089 ± 0.01	0.077 ± 0.01	0.101 ± 0.01	0.057 ± 0.02	0.102 ± 0.00
5% PP	0.084 ± 0.02	0.065 ± 0.01	0.105 ± 0.01	0.070 ± 0.02	0.069 ± 0.01
10% PP	0.088 ± 0.02	0.062 ± 0.00	0.092 ± 0.01	0.066 ± 0.02	0.090 ± 0.00
15% PP	0.081 ± 0.02	0.065 ± 0.01	0.089 ± 0.01	0.084 ± 0.01	0.093 ± 0.01

[a] Within each storage day, mean values (±standard deviation) in the same column are not significantly different ($P > 0.05$).

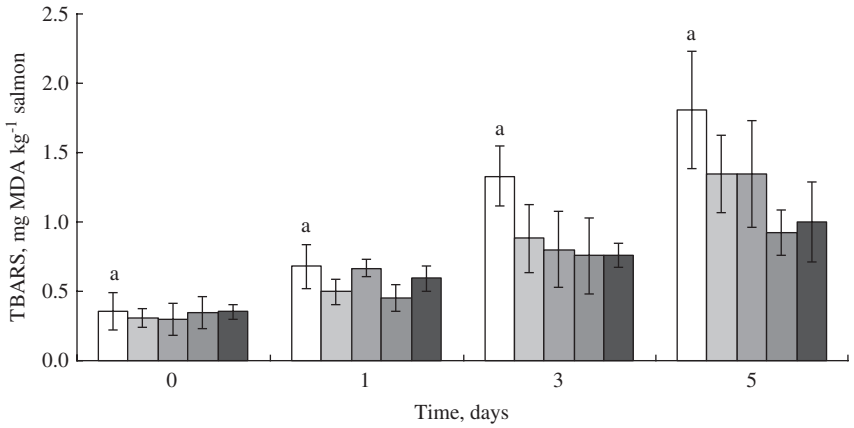

Figure 1. Effect of dietary *Palmaria palmata* (PP) on lipid oxidation (TBARS) in cooked salmon fillets ($n = 3$) stored aerobically for up to 5 days at 4 °C: □, PC; ▨, 0% PP; ▩, 5% PP; ▩, 10% PP; ■, 15% PP. [a]Within each storage day, mean values (±standard deviation) are not significantly different ($P > 0.05$).

The increase in $-a^*$ (greenness) and b^* (yellowness) colour measurements with increasing dietary *P. palmata* concentration indicated deposition of colour pigments in the muscle. The antioxidant potential *in vitro* of both fat- and water-soluble compounds (lutein, β-carotene, phycobiliproteins, chlorophyll) of *P. palmata* is well documented.[40] Additionally, the antioxidant activity of extracts from *P. palmata* has been reported using a number of *in vitro* antioxidant assays (deoxyribose assay, DPPH, ABTS, TBARS, reducing activity, transition metal ion chelation).[18] Previous studies indicated that dietary antioxidants such as α-tocopherol fed to Atlantic halibut enhanced lipid stability in fish muscle, with significantly lower levels of lipid oxidation reported in fish fillets.[41] Despite the antioxidant potential of *P. palmata*, no increase in lipid stability of the salmon fillets was observed in the present study.

To further examine the antioxidant potential of compounds from *P. palmata* deposited in the fish muscle, raw salmon fillet homogenates were subjected to iron ($FeCl_3$)/ascorbate-induced lipid oxidation. Following $FeCl_3$ and ascorbic acid addition, lipid oxidation increased in all tissue homogenates over the 24 h storage period. No significant differences were observed between PC and all PP treatments (0–15%), with mean values ranging from 0.21 to 1.77 mg MDA kg^{-1} salmon (Fig. 2). Similarly, astaxanthin deposited in farmed Atlantic salmon fillets demonstrated no significant antioxidant activity under standardised conditions of accelerated oxidation. Although no effect of astaxanthin as an antioxidant in fish muscle was measured, it was concluded that retention of the carotenoid may offer other beneficial biological effects such as enhanced immune response, inhibition of mutagensis and reduction of photo-induced nuclear damage in cells and tissues.[21,42]

Under conditions employed in the present study (fresh fillets stored in MAPs, cooked fillets stored aerobically, fresh fillet homogenates subjected to iron/ascorbate induced oxidation), levels of lipid oxidation in salmon fillets were unaffected by dietary *P. palmata*. However, *in vitro* antioxidant activity of *P. palmata* has been reported using extracts with purified bioactive components and at concentrations greater than those tested in the present study. The antioxidant activity of carotenoids and other compounds *in vivo* depends on numerous factors such as form, concentration, cellular distribution and interaction with other components present in the muscle tissue.[4] In the present study, the lack of antioxidant activity in salmon fillets may have been due to the form or concentration of components deposited in the muscle.

Although retention of pigments from *P. palmata* did not offer an increase in lipid stability to the salmon fillets under the conditions tested, carotenoids are still necessary for salmon growth and development.[6] Natural pigments retained in salmon fillets may offer added benefit to consumers. Lutein, for example, has been linked to maintenance of normal visual function in the human eye macula.[43] Further studies would need to examine the bioavailability of colour pigments deposited in the fish muscle.

Microbiology of fresh salmon fillets

Mesophilic and psychrotrophic total viable counts (TVCs) increased during storage of fresh salmon fillets in MAPs, ranging from ~1.9 to 9.1 $\log_{10}$ CFU g^{-1} and from ~2.3 to 9.0 $\log_{10}$ CFU g^{-1} respectively (Table 5). Mesophilic counts obtained were similar to previously reported values for fresh salmon fillets stored at 4 °C under normal conditions, where initial colony counts ranged from

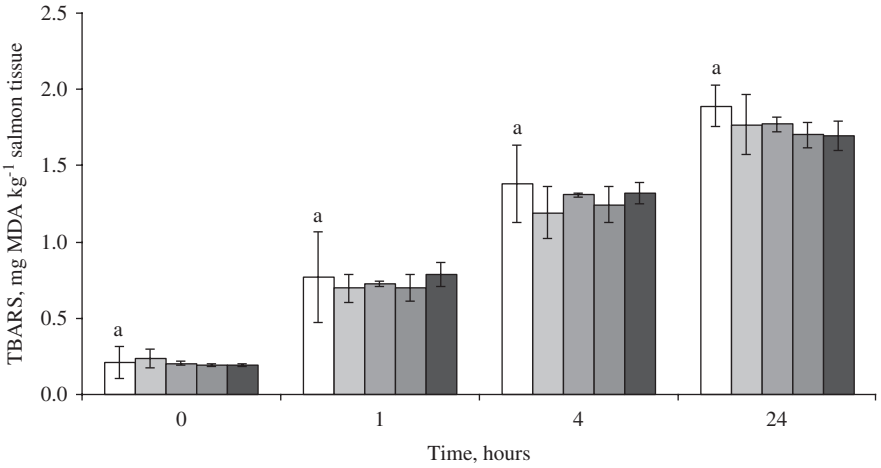

Figure 2. Effect of dietary *Palmaria palmata* (PP) on lipid oxidation (TBARS) in salmon fillet homogenates ($n = 3$) containing equimolar $FeCl_3$/ascorbate (45 μmol L^{-1}) and stored for 24 °h at 4°C: □, PC; ■, 0% PP; ■, 5% PP; ■, 10% PP; ■, 15% PP. [a]Within each storage time, mean values (±standard deviation) are not significantly different ($P > 0.05$).

Table 5. Effect of dietary *Palmaria palmata* (PP) on microbial status (mesophilic and psychrotrophic, $\log_{10}$ CFU g^{-1} salmon) of fresh salmon fillets ($n = 3$) stored in modified atmosphere packs (60% N_2/40% CO_2) for up to 15 days at 4 °C

		Storage time at 4 °C (days)				
Treatment	Temperature (°C)	1	3	7	12	15
PC	30	1.93 ± 0.40[a]	2.69 ± 0.64[a]	6.83 ± 0.98[a]	8.39 ± 0.62[a]	9.27 ± 0.59[a]
0% PP		2.03 ± 0.35	1.66 ± 1.52	5.41 ± 1.00	8.38 ± 0.33	9.01 ± 1.39
5% PP		2.35 ± 0.37	2.63 ± 0.49	5.98 ± 1.22	7.74 ± 1.19	8.87 ± 0.66
10% PP		1.78 ± 1.56	2.81 ± 0.36	5.67 ± 0.76	7.37 ± 1.07	9.38 ± 0.27
15% PP		1.43 ± 1.25	1.49 ± 1.29	6.55 ± 0.92	8.40 ± 0.46	9.14 ± 0.22
PC	4	1.96 ± 1.71[a]	3.59 ± 1.01[a]	6.57 ± 0.75[a]	8.61 ± 0.54[a]	8.89 ± 0.56[a]
0% PP		1.90 ± 1.65	3.26 ± 0.73	5.35 ± 0.96	8.55 ± 0.28	9.44 ± 1.30
5% PP		1.13 ± 1.96	2.37 ± 2.12	5.86 ± 0.90	7.54 ± 1.34	8.69 ± 0.91
10% PP		3.43 ± 0.43	2.28 ± 2.10	5.56 ± 0.88	7.70 ± 0.70	8.89 ± 0.12
15% PP		3.18 ± 0.31	2.48 ± 2.15	6.60 ± 1.03	8.59 ± 0.48	9.18 ± 0.22

[a] Within each parameter and storage day, mean values (±standard deviation) in the same column are not significantly different ($P > 0.05$).

2 to 5 $\log_{10}$ CFU g^{-1} on day 0 and end of shelf-life was determined as colony counts > 7 $\log_{10}$ CFU g^{-1}.[44] In the present study, on day 12, all salmon fillets had mesophilic and psychrotrophic counts of ~8 $\log_{10}$ CFU g^{-1} and were considered unsafe for human consumption. The TVCs for 5, 10 and 15% PP were similar ($P > 0.05$) for all treatments, on each storage day, compared with 0% PP and PC, indicating that dietary *P. palmata* did not result in salmon fillets with enhanced antimicrobial stability.

Previously reported studies indicate that the antimicrobial activity of seaweeds is dependant on concentration, solvent use and extraction method as well as the sensitivity of the methods used to determine antimicrobial activity.[45,46] Cox *et al.*[45] reported that methanolic extracts of *P. palmata* were moderately effective against *Listeria monocytogenes* (62.09%) and showed weak activity against *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Salmonella abony* (39.28, 19.22 and 2.21% respectively) using the microtitre method. Ethanolic extracts of *P. palmata* increased inhibition of bacteria to 100 and 93.89% against *E. faecalis* and *P. aeruginosa* respectively. However, no inhibition of five fish pathogenic bacteria strains was found using *P. palmata* extracts in the standard agar plate diffusion assay.[47] *In vitro* studies demonstrated antimicrobial activity through the use refined or purified *P. palmata* extracts; however, to date, no scientific literature demonstrating antibacterial activity of seaweeds or seaweed extracts in food products exists. In the present study, the lack of antimicrobial activity in the salmon fillets may have been due to the purity or level (5–15%) of *P. palmata* incorporated in the salmon feed.

Sensory evaluation of fresh and cooked salmon fillets

In 'visual' sensory analysis, fillets from fresh salmon fed astaxanthin (PC) were significantly positively correlated with pinkness, purchasing appeal and overall acceptability ($P < 0.001$) on days 1 and 7 of storage (Table 6). On days 1 and 7 of storage, salmon fed at all PP levels (0, 5, 10 and 15%) were significantly negatively correlated with pinkness, purchasing appeal and overall acceptability. The observations of the panellists were in agreement with instrumental a^* (redness) values, where PC had the largest a^* values compared with all PP treatments. Proximate composition indicated that moisture (~74–76 g per 100 g) was similar across all treatments (Table 2) and dietary *P. palmata* did not influence drip loss on days 1 and 7 of storage. Randell *et al.*[39] reported that drip loss was low (~1.5%) in salmon stored in MAPs (60% N_2/40% CO_2).

Table 6. Significance of regression coefficients (ANOVA values) of sensory analysis scores for fresh and cooked salmon fillets ($n = 3$) as derived by jack-knife uncertainty testing

	Treatment/storage time (days)									
	PC		0% PP		5% PP		10% PP		15% PP	
Parameter	1	7	1	7	1	7	1	7	1	7
Visual sensory analysis										
Pinkness	0.00***	−0.02*	−0.10	0.00***	−0.00***	−0.00***	−0.05*	−0.003**	−0.02*	−0.00***
Whiteness	−0.00***	0.02*	0.10	−0.00***	0.00***	0.00***	0.04*	0.003**	0.01*	0.00***
Drip	−0.28	0.37	−0.55	0.53	0.28	−0.53	0.32	−0.56	0.29	−0.52
Purchasing appeal	0.00***	−0.04*	−0.10	0.00***	−0.00***	−0.00***	−0.06	−0.003**	−0.03*	−0.00***
Overall acceptability	0.00***	−0.03*	−0.10	0.00***	−0.00***	−0.00***	−0.05*	−0.003**	−0.02*	−0.00***
Eating quality sensory analysis										
Colour	0.00***	0.05*	−0.11	−0.00***	−0.00***	−0.00***	−0.07	−0.003**	−0.03*	−0.00***
Texture	0.35	−0.39	−0.35	0.18	−0.36	−0.20	−0.40	−0.21	−0.34	−0.16
Odour	−0.68	0.70	−0.39	0.40	0.68	−0.43	0.69	−0.40	0.69	−0.42
Oxidation flavour	−0.24	0.33	0.39	−0.33	0.24	0.35	0.31	0.36	0.28	0.32
Overall acceptability	0.00***	−0.06	−0.13	0.00***	0.00***	0.00***	−0.08	−0.02*	−0.03*	0.00***

*95% significance, $P < 0.05$;
**99% significance, $P < 0.01$;
***99.9% significance, $P < 0.001$.

In 'eating quality' sensory analysis, PC was significantly positively correlated with cooked salmon fillet colour while 5% PP was significantly negatively correlated with fillet colour ($P < 0.001$) on both analysis days. Sensory panellists were able to distinguish between controls and salmon fed PP based on the colour of the cooked salmon fillets. Texture, odour and oxidation flavour in cooked salmon fillets were not significantly influenced by dietary PP. The ability of panellists to detect no oxidation/off-flavour in the salmon fillets is in agreement with the low levels of lipid oxidation (TBARS) found. On days 1 and 7, salmon fed 5% PP were significantly positively correlated ($P < 0.001$) with overall acceptability (Table 6). In a previously reported study, feeding red tilapia (*Oreochromis niloticus*) with *Spirulina platensis* as the sole source of fish feed did not impact on the taste and smell parameters of the cooked fish fillets.[48] In the present study, salmon fed low levels of PP (5%) showed increased overall acceptability, compared with controls, by sensory panellists despite their ability to distinguish between fillets based on colour. Therefore *P. palmata* incorporated in salmon feed at low levels (5%) may offer enhancement of overall acceptability without negatively impacting on texture, odour or oxidation flavour.

CONCLUSIONS

Salmon fillet surface colour was enhanced with a yellow/orange colour due to deposition of *P. palmata* pigments. Owing to consumers' growing preference for additives from natural sources, *P. palmata* may prove to be a natural pigment alternative when incorporated into salmon feed and have application in the development of novel functional salmon products. Further research is necessary to investigate if more refined *P. palmata* extracts increase lipid stability. *Palmaria palmata* enhanced overall 'eating quality' acceptability of salmon fillets with comparable product quality (texture, odour and oxidation flavour) and proximate composition to Atlantic salmon (*S. salar*) fed synthetic astaxanthin. Therefore *P. palmata* may be considered as a replacement functional ingredient for farmed Atlantic salmon feed. Further studies will need to examine the use of *P. palmata* with other carotenoid sources to adequately reach final red/pink flesh levels acceptable to consumers.

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Addition of seaweed (*Laminaria digitata*) extracts containing laminarin and fucoidan to porcine diets: Influence on the quality and shelf-life of fresh pork

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ABSTRACT

A seaweed extract containing laminarin (L) and fucoidan (F) (L/F) was manufactured from brown seaweed (*Laminaria digitata*) in spray-dried (L/F-SD) and wet (L/F-WS) forms. The effect of supplementation of pig diets with L/F-SD and L/F-WS (L, 500 mg/kg feed; F, 420 mg/kg feed) for 21 days pre-slaughter, on quality indices of fresh *M. longissimus dorsi* (LD) steaks was examined. Susceptibility of porcine liver, heart, kidney and lung tissue homogenates to iron-induced (1 mM $FeSO_4$) lipid oxidation was also investigated. Dietary supplementation with L/F did not increase plasma total antioxidant status (TAS). In LD steaks stored in modified atmosphere packs (80% O_2:20% CO_2) (MAP) for up to 15 days at 4 °C, muscle pH, surface colour (CIE '*L**' lightness, '*a**' redness and '*b**' yellowness values) and microbiology (psychrotrophic and mesophilic counts, log CFU/g pork) were unaffected by dietary L/F. In general, levels of lipid oxidation (TBARS, mg MDA (malondialdehyde)/kg pork) followed the order: C > LF-SD > L/F-WS. A statistically significant reduction in lipid oxidation ($P<0.05$) was observed in LD steaks from 75% of pigs ($n=6$) fed with L/F-WS compared to controls. Iron-induced lipid oxidation increased in liver, heart, kidney and lung tissue homogenates over the 24 h storage period and dietary L/F-WS reduced lipid oxidation to the greatest extent in liver tissue homogenates. Results demonstrate potential for the incorporation of marine-derived bioactive antioxidant components into muscle foods *via* the animal's diet.

1. Introduction

Meat and meat products are considered to be a vital component of a healthy diet and important sources of protein, vitamins, minerals and trace elements. In recent years, consumer confidence in meat has been undermined by a number of health concerns related to meat consumption, for example, saturated fat and cholesterol and the associated risk of heart disease, cancer, obesity. Increased consumer demand for healthier meat and meat products, with reduced fat levels, cholesterol, sodium chloride, nitrite, enhanced fatty acid profile and containing health promoting/enhancing ingredients has led to the development of functional meat and meat products (Toldrá & Reig, 2011). The mode of action of functional foods is based on the use of functional ingredients which exert a range of bioactive properties such as antioxidant, anti-inflammatory, anti-cancer and anti-diabetic activities. As concerns regarding the safety and toxicity of synthetic antioxidants grow, the functional properties of many plant extracts have been investigated for their potential use as novel functional ingredients/nutraceuticals.

A number of strategies exist, whereby antioxidants may be incorporated into meat and meat products in order to facilitate the development of functional meats with enhanced health-promoting properties (Khan et al., 2011). Strategies include the supplementation of animal diets with antioxidant compounds or the direct addition of antioxidants to processed meat products. Previous research has focused on dietary supplementation studies, for example, vitamin E (α-tocopheryl acetate), significantly improved the lipid stability of fresh pork (Asghar et al., 1991; Lanari, Schaefer, & Scheller, 1995; Monahan, Asghar, Gray, Buckley, & Morrissey, 1994). Similarly, antioxidant plant extracts such as tea catechins reduced lipid oxidation in poultry muscle (Tang, Kerry, Sheehan, Buckley, & Morrissey, 2001). Dietary supplementation of oregano oleoresins into porcine diets also reduced lipid oxidation in minced pork (Janz, Morel, Wilkinson, & Purchas, 2007).

Direct addition of plant extracts also reduces lipid oxidation in muscle foods. Carob fruit extracts were found to exhibit antioxidant activity in cooked pork muscle homogenates when added at levels which previously demonstrated bioactivity (reduction in total and LDL cholesterol) in hypercholesterolemic human subjects (Bastida et al., 2009). Lipid oxidation was reduced in pork meat homogenates as a result of the addition cloudberry, beetroot and willow herb (Rey, Hopia, Kivikari, & Kahkonen, 2005). Addition of tea catechins, rosemary and sage also reduced levels of lipid oxidation in fresh pork patties (McCarthy, Kerry, Kerry, Lynch, & Buckley, 2001).

Macroalgae (seaweed) and are a potential source of natural antioxidants. Although the nutrient content of seaweed vary with species,

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geographical location, season and temperature, most contain significant quantities of carbohydrates (polysaccharides and dietary fibre), protein (essential amino acids), lipids (phospholipids), vitamins (ascorbic acid and beta carotene) and minerals (calcium, iron, and potassium) (Gupta & Abu-Ghannam, 2011). Brown seaweeds contain polyphenolic antioxidant compounds such as phlorotannins (phloroglucinol and eckol), catechins (catechin, epigallocatechin, and epigallocatechin gallate), tocopherols (α-, γ-, and δ-tocopherols), ascorbic acid and carotenoids (α- and β- carotenes and fucoxanthin) (Zubia et al., 2009). The vast range of compounds present increases potential for the use of seaweed and/or extracts in the development of functional foods.

Brown seaweeds are rich in polysaccharides (soluble dietary fibre), the most abundant of which are laminarin, fucoidan and alginic acid. The chemical structure of laminarin (β-glucan) consists mainly of a linear β-(1,3)-linked glucose backbone with some random β-(1,6)-linked glucose side chains depending on the variety of seaweed used for extraction (O'Doherty, Dillon, Figat, Callan, & Sweeney, 2010). Structurally, fucoidan (fucan) is a sulphated polysaccharide containing L-fucose (Costa et al., 2010). Seaweed polysaccharides, including laminarin and fucoidan are reported to possess antioxidant (Heo, Park, Lee, & Jeon, 2005), antitumour, antiviral, and antibacterial activities (Costa et al., 2010; O'Doherty et al., 2010; Zubia et al., 2009).

Scientific studies on the potential for incorporating health promoting bioactive compounds derived from seaweed into muscle foods, *via* supplementation of animal diets, are limited and merit investigation. In addition to deposition of bioactive compounds in muscle tissues of meat producing animals, dietary bioactive compounds also demonstrate potential to improve animal health and welfare. Supplementation of pig diets with laminarin and fucoidan (isolated from *Laminaria digitata*) has previously been shown to improve growth performance and gut health in pigs (O'Doherty et al., 2010).

The objective of this study was to assess the effect of dietary supplementation of porcine diets with a seaweed extract containing laminarin and fucoidan (L/F), isolated from *L. digitata*, on the plasma antioxidant status, muscle pH, colour, lipid oxidation and microbiology of fresh *M. longissimus dorsi* (LD) steaks stored in modified atmosphere packs (MAP) at 4 °C. The influence of dietary L/F on iron-induced lipid oxidation in porcine liver, heart, kidney and lung tissue homogenates was also examined.

2. Materials and methods

2.1. Reagents

All chemicals used were 'AnalaR' grade obtained from Sigma-Aldrich Ireland Ltd., Arklow, Co. Wicklow, Ireland, Oxoid Ltd., Basingstoke, Hampshire, England and Merck KGaA, Darmstadt, Germany. The total antioxidant status (TAS) Randox-Trolox kit was obtained from Randox Laboratories Ltd., Co. Antrim, UK. A seaweed extract (L/F), containing laminarin (L) and fucoidan (F) was manufactured by Bioatlantis, Tralee, Co. Kerry, Ireland. The extract isolated from brown seaweed (*L. digitata*), harvested in Ireland, was prepared using an acid extraction technique, details of which are confidential.

2.2. Animals and diets

Twenty four pigs (Large White × Landrace cross consisting of 12 males and 12 females) (average live weight ~ 14.51 kg) were randomly assigned to one of three treatments (n = 8) and fed *ad libitum* for 21 days pre-slaughter following a completely randomised experimental design. The control group (Control) were fed with a basal diet (Table 1). The second group were fed with the basal diet plus a spray-dried seaweed extract containing laminarin and fucoidan (L/F-SD) at an inclusion rate of 5.37 kg/tonne of feed. The third group were fed with the basal diet plus a wet formulation of the seaweed extract containing laminarin and fucoidan (L/F-WS) at an inclusion rate of 26.3 kg/tonne of feed. Inclusion rates are based on the laminarin and fucoidan content of the spray-dried (L/F-SD) and wet (L/F-WS) seaweed extracts. Therefore both treatment groups received diets containing L, 500 mg/kg feed and F, 420 mg/kg feed.

The composition of the experimental diet and the seaweed extracts containing L/F are outlined in Tables 1 and 2, respectively. Dry matter, crude protein, neutral detergent fibre, ash and gross energy analysis of the experimental diet was carried out as described by Leonard, Sweeney, Bahar, Lynch, and O'Doherty (2011). The laminarin content (%) of the spray-dried (L/F-SD) and wet (L/F-WS) seaweed extracts was measured using a commercial assay kit (Megazyme International Ireland, Bray, Co. Wicklow, Ireland). Fucoidan levels (%) in L/F-SD and L/F-WS were determined as described by Usov, Smirnova, and Klochkova (2001).

The animals were housed individually (1.68 m × 1.22 m slatted pens) at Lyons Research Farm, University College Dublin, Newcastle, Co. Dublin, Ireland and fed *ad libitum* from hopper style feeders. Water was supplied *ad libitum* from individual nipple drinkers. The average daily feed intake was 1.8 kg/day for the control and L/F-WS groups, and 1.9 kg/day for the L/F-SD group (SEM 0.09). The ambient environmental temperature within the houses was thermostatically controlled and maintained at 22 °C. The pigs were slaughtered at the end of the feeding period *via* euthanol injection (pentobarbitone sodium patent blue) (injection rate: 1 ml/1.4 kg live weight) by veterinary personnel. A blood sample for plasma analysis was taken immediately following injection. The mean slaughter weight was 29.7 kg. The liver and kidneys were removed and frozen in a blast freezer. Carcasses and organs were transported at 4 °C to the School of Food and Nutritional Sciences at University College Cork, Ireland where they were hung at 2 °C for 24 h. Liver, heart, kidney and lung tissues were placed in vacuum pack bags (composed of polyamide and a polyethylene sealing layer), vacuum packed and stored at −18 °C for four months prior to analysis. Blood samples were centrifuged to separate the plasma fractions and stored at −20 °C prior to analysis.

Table 1
Composition and chemical analysis of the experimental diet (g/kg, unless otherwise indicated).

Composition	Basal diet[a]
Wheat	660.40
Soya-bean meal	260.00
Soya oil	24.80
Minerals and vitamins[b]	23.00
Lysine HCL	3.40
L-Threonine	1.30
DL-Methionine	0.80
Analysed composition	
Dry matter	887.70
Crude protein (N*6.25)	186.60
Neutral detergent fibre	115.30
Ash	46.50
Gross energy (MJ/kg)	17.30
Calcium[c]	6.90
Phosphorus[c]	4.35
Lysine[c]	10.00
Methionine and cysteine[c]	6.00
Threonine[c]	6.50
Tryptophan[c]	1.80

[a] Control group — basal diet; L/F-SD — basal diet supplemented with 5.37 kg/tonne of spray dried seaweed extract containing L/F and 20.9 kg water; L/F-WS — basal diet supplemented with 26.3 kg/tonne of wet seaweed extract containing L/F.

[b] Vitamin and mineral inclusion (per kg diet): 3 mg retinol, 0.05 mg cholecalciferol, 40 mg α-tocopherol, 25 mg copper as copper sulphate, 100 mg iron as iron sulphate, 100 mg zinc as zinc oxide, 0.3 mg selenium as sodium selenite, 25 mg manganese as manganese oxide and 0.2 mg iodine as calcium iodate on a calcium sulphate/calcium carbonate carrier.

[c] Calculated from tabulated nutritional composition (Sauvant, Perez, & Tran, 2004).

Table 2
Composition of the seaweed extracts containing laminarin and fucoidan.

	L/F-SD	L/F-WS
% Total solids	94.03	18.5
% Ash[a]	68.6	13.4
% Protein	0.64	0.12
% Laminarin	9.3	1.9
% Fucoidan	7.8	1.6
% Mannitol	8.3	1.62
Inclusion rate (per tonne feed)	5.37 kg	26.3 kg

[a] The ash content of the seaweed extract was 15 g/kg DM Ca, 10 g/kg DM Na, 10 g/kg DM K, 10 g/kg DM S, 250 mg/kg DM iodine, 250 mg/kg DM Fe, 20 mg/kg DM Cu and 50 mg/kg DM Zn.

2.3. Plasma total antioxidant status

Blood samples (10 ml) were collected by jugular veni-puncture using vacutainers containing lithium/heparin as anti-coagulant (Becton Dickinson, Rutherford, NJ, USA) from all animals immediately after slaughter. The blood was centrifuged (Beckman J2-21, Beckman Instruments Inc., CA, USA) at 4720 g for 20 min at 4 °C. The plasma layer was removed from the red blood cell layer and stored at −18 °C for subsequent analysis.

The total antioxidant status (TAS) of porcine blood plasma was measured according to the manufacturers' instructions. Plasma (20 μl) was added to 1 ml chromogen (metmyoglobin/ABTS®) and mixed thoroughly. Tubes were placed in a water bath at 37 °C for 10 min. An initial absorbance reading of the coloured complex was recorded after 10 min using a spectrophotometer (Cary 300 Bio, UV–vis spectrophotometer, Varian Instruments, CA, USA) at 600 nm against a blank containing all reagents and double de-ionised water. Tubes were returned to the water bath and 200 μl of substrate (hydrogen peroxide in stabilised form) was added. A second absorbance measurement of the colour complex was recorded 3 min following substrate addition. The antioxidant activity was calculated using the following equation:

$$mmol/l = Factor \times (\Delta A\ Blank - \Delta A\ Sample)$$

$$Factor = (2.04\ mmol/l)/(\Delta A\ Blank - \Delta A\ Standard)$$

Total antioxidant status (TAS) was expressed as mmol of trolox equivalent antioxidant capacity (TEAC)/L plasma.

2.4. Pork processing and packaging

Following storage at 2 °C for 24 h, *M. longissimus dorsi* was excised from each carcass, vacuum packed and stored at 2 °C for 24 h. The heart and lungs were removed from each carcass, vacuum packed and stored at −18 °C with the previously blast-frozen liver and kidney tissues.

M. longissimus dorsi (LD) were cut into steaks (~1 in. thickness, ~45 g portion), placed in low oxygen permeable (<1 cm^3/m^2/24 h at STP) polystyrene/ethylvinylalcohol (EVOH)/polyethylene (PE) trays and flushed with 80% O_2:20% CO_2 (modified atmosphere packs, MAP) using a vacuum-sealing unit (VS 100, Gustav Müller & Co. KG, Homburg, Germany) equipped with a gas mixer (Witt-Gasetechnik GmbH & Co. KG, Witten, Germany). Trays were covered and heat-sealed using a low oxygen permeable (3 cm^3/m^2/24 h at STP) laminated barrier film with a polyolefin heat sealable layer. LD steaks in MAP were stored for up to 15 days under fluorescent lighting (660 lx) at 4 °C. The gas atmosphere in the MAP was checked using a CheckMate 9900 (PBI-DanSensor, Denmark). Immediately after gas flushing, MAP contained 70.18 ± 0.33% O_2 and 23.14 ± 0.31% CO_2. The average gas composition in MAP at the end of the 14 day storage period was 68.43 ± 1.02% O_2 and 23.88% ± 0.59% CO_2.

2.5. Measurement of pH

Pork samples (10 g) were homogenised for 1 min at 24,000 rpm in 90 ml distilled water using an Ultra Turrax T25 homogeniser (Janke and Kunkel, IKA-Labortechnik, GmbH and Co., Staufen, Germany). The pH of the pork homogenates was measured at 20 °C using a pH meter (Seven Easy portable, Mettler-Toledo GmbH, Schweizenbach, Switzerland). The pH of LD steaks was recorded on days 1, 4, 7, 11 and 14 of storage.

2.6. Colour Measurement

The surface colour was measured using a Minolta CR-300 Chroma Meter (Minolta Camera, Co., Osaka, Japan). The chroma meter consisted of a measuring head (CR-300), with an 8 mm diameter measuring area, a 2° standard observer, and a data processor (DP-301). The chroma meter was calibrated on the CIE LAB colour space system using a white tile (D_c: $L = 97.79$, $a = -0.11$, $b = 2.69$). The 'L^*' value represents lightness and 'a^*' and 'b^*' values represent redness and yellowness, respectively. Colour measurements of LD steaks were recorded on days 1, 4, 7, 11 and 14 of storage.

2.7. Measurement of lipid oxidation

Lipid oxidation was measured using the 2-thiobarbituric acid assay of Siu and Draper (1978). The malondialdehyde content was calculated using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$. Results were expressed as 2-thiobarbituric acid reactive substances (TBARS) in mg malondialdehyde (MDA)/kg pork. Lipid oxidation in LD steaks was measured on days 1, 4, 7, 11 and 14 of storage.

2.8. Microbiological analysis

Pork (10 g) was transferred into stomacher bags, diluted with 90 ml of maximum recovery diluent and stomached for 3 min (Steward Stomacher 400 Lab Blender, London, UK) resulting in a 10^{-1} dilution used for analysis. Serial dilutions were prepared and 0.1 ml aliquots from each dilution were plated onto standard plate count agar (PCA) (Oxoid Ltd.). The plates were incubated at 30 °C for 48 h and at 4 °C for 10 days to determine mesophilic and psychrotrophic counts, respectively. Microbiological analysis of LD steaks was carried out on days 1, 5, 8, 12 and 15 of storage. Results were expressed as $\log_{10}$CFU (colony forming units)/g pork.

2.9. Preparation of liver, heart, kidney and lung tissue homogenates

Liver, heart, kidney and lung tissue homogenates (25%) were prepared by homogenising 20 g tissue in 60 ml 0.12 M KCl 5 mM histidine (pH 5.5) using an Ultra Turrax T25 homogeniser at 24,000 rpm for 3 min. Lipid oxidation in 30 ml tissue homogenate samples, held in 150 ml beakers at 4 °C, was initiated by the addition of 1 mM $FeSO_4$. Homogenates without $FeSO_4$ were run simultaneously as controls. Lipid oxidation was measured immediately (time 0) and after 24 h of storage at 4 °C.

2.9.1. Measurement of lipid oxidation in organ tissue homogenates

A modification of the 2-thiobarbituric acid (TBA) assay of Siu and Draper (1978) was used to measure lipid oxidation in liver, heart, kidney and lung tissue homogenates. Homogenate samples (4 ml) were added to 4 ml 10% trichloroacetic acid (TCA). The samples were mixed using a vortex mixer and the precipitate formed was removed by filtering through Whatman No. 1 filter paper. In a screw cap test tube, 4 ml of clear supernatant was added to 1 ml 0.06 M 2-thobarbituric acid (TBA). The tubes were incubated at 80 °C for 90 min and the absorbance of the resulting coloured complex was measured using a spectrophotometer at 532 nm against a blank containing all reagents and distilled water

instead of the filtrate. Tubes containing supernatant from filtered liver homogenates and 10% TCA, incubated with TBA reagent, were centrifuged at 1000 g, prior to absorbance measurements to pellet a precipitate formed during the heating step. The malondialdehyde content was calculated using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$. Results were expressed as TBA reactive substances (TBARS) in mg malondialdehyde (MDA)/kg organ tissue.

2.10. Statistical analysis

All analyses were performed in duplicate. Mean sample values (n = 8) for each of the three treatment groups (Control, L/F-SD and L/F-WS) were subjected to statistical analysis. A full repeated measures ANOVA was conducted to investigate the effects of dietary L/F form (spray-dried (L/F-SD) and wet (L/F-WS) forms), time and their interactions. Dietary L/F form represented the 'between-subjects' factor and the effect of time was measured using the 'within-subjects' factor. Tukey's test was used to adjust for multiple comparisons between treatment means. The analysis was carried out using the SPSS 18.0 for Windows (SPSS, Chicago, IL, USA) software package.

3. Results and discussion

3.1. Plasma total antioxidant status and muscle pH

In previously reported studies, antioxidant compounds have been detected in the plasma of animals and humans following ingestion. Detection of antioxidant compounds in blood plasma is a useful indicator of the fate of supplemental extracts and suggests that compounds or their metabolites are readily bio-available for potential uptake into muscle tissues. The consumption of foods rich in phenolic antioxidants (strawberries, spinach or red wine) has been found to significantly increase the antioxidant status of plasma in humans (ORAC, TEAC and FRAP assays) (Cao, Russell, Lischner, & Prior, 1998). In rat plasma elevated tea catechin concentrations have been reported following oral administration of a single dose (500 mg/kg body weight) (Nakagawa & Miyazawa, 1997). Antioxidants such as vitamin E (α-tocopherol) have been detected in porcine plasma with levels increasing 2.5-fold after approximately 7 days of supplementation (200 mg/kg feed) (Morrissey, Buckley, Sisk, Lynch, & Sheehy, 1996).

In the present study, analysis of porcine plasma for laminarin and fucoidan was not carried out due to the complex structural properties of the seaweed polysaccharides. In order to assess the effectiveness of dietary supplementation with L/F, the total antioxidant status (TAS) of porcine plasma was determined. The average TAS was 0.55, 0.67 and 0.60 mmol TEAC/L plasma for the control group, L/F-SD and L/F-WS, respectively. Porcine plasma TAS was not significantly affected by L/F supplementation compared to the control group. Gladine, Morand, Rock, Bauchart, and Durand (2007a) reported no increase in plasma TAS in rats fed with plant extracts (rosemary, grape, citrus and marigold, 0.5 g/kg diet for three weeks), however a significant reduction in plasma lipid oxidation (malondialdehyde (MDA)) was observed. While laminarin and fucoidan have strong antioxidant capacities *in vitro* (Heo et al., 2005; Wang, Zhang, Zhang, Song, & Li, 2010), to date limited research exists to support the contention that consumption of polysaccharides augments plasma antioxidant levels *in vivo*. Gladine et al. (2007a) reported no correlation between *in vitro* and *in vivo* antioxidant capacity of plant extracts rich in polyphenols and further suggested that bioavailability efficiency and TAS are more a general indicator of plasma oxidative stability rather than specifically reflecting lipid susceptibility to oxidation.

The reason for the lack of an effect of dietary L/F on the TAS of porcine plasma is unclear. In a previously reported study which examined the absorption of dextran sulphate (branched glucan) (MW about 8000), administered orally in a short-term (single dose, 4 g/day for 5 days) and long term (1 g, 4 times/day for 29 to 335 days) study, dextran sulphate was detected in high concentrations in endothelial cells and at low levels in plasma as early as 2.4 min after administration. It was concluded that dextran sulphate was extensively absorbed and rapidly incorporated into the endothelium therefore plasma levels were negligible (Hiebert, Wice, Jaques, Williams, & Conly, 1999). By contrast, a single dose (0.1 ml/10 g of body weight) of α-D-glucans from fungi (*Tricholoma matsutake*) administered to mice, resulted in detection of α-D-glucans in mice plasma 16 h following oral administration. Maximum α-D-glucans levels were detected by 24 h, after which, levels declined up to 48 h (Hoshi, Iijima, Ishihara, Yasuhara, & Matsunaga, 2008). Previous literature reported that distribution of glucans in the body is affected by various factors, such as molecular weight, particle size, fine structure, charge, association, and susceptibility to enzymatic hydrolysis (Hiebert et al., 1999). In the present study, pigs were fed up to 3 h prior to slaughter, therefore rapid absorption of L/F by the GI tract may account for the lack of an effect of dietary L/F on the total antioxidant status (TAS) of porcine plasma.

The pH of fresh LD muscle ranged from 5.90 to 5.19 over the 15 day storage period and was unaffected by L/F supplementation of porcine diets. pH values reported are comparable to previously reported values (5.8–5.4) for post-mortem muscle (Faustman & Cassens, 1990).

3.2. Colour stability of fresh LD muscle

The surface lightness 'L^*' and yellowness 'b^*' values increased in fresh LD muscle over the 14 day storage period. Surface 'a^*' redness values decreased as a function of storage time (Table 3). Each colour parameter was not affected by L/F supplementation. Many studies have provided evidence that pigment (oxymyoglobin) oxidation and lipid oxidation are closely interrelated where an increase in one results in a similar increase in the other. This is potentially due to decreased dissolved oxygen levels or radicals produced as a result of the lipid oxidation process (O'Grady, Monahan, & Brunton, 2001). Levels of lipid oxidation were relatively low over the 14 day storage period. Therefore protection against pigment oxidation due to L/F supplementation, mediated through a reduction in lipid oxidation, was not evident in the present study.

While some previously reported studies indicate that colour stability of pork muscles may be improved when antioxidants are incorporated into animal diets, others demonstrate no effect of dietary antioxidants on fresh pork colour stability. For example, in pigs fed with vitamin E (α-tocopheryl acetate), some studies report positive benefits of feeding vitamin E on pork colour stability, while others report no beneficial effects on fresh pork colour (Asghar et al., 1991; Houben, Eikelenboom, & Hoving-Bolink, 1998; Lanari et al., 1995; Monahan et al., 1994). Similar to results reported in the present study, oat-based diets containing β-glucans (1.6, 2.1, 3.3 or 4.1%) did not influence the colour stability of porcine *longissimus* muscle (Fortin, Robertson, Kibite, & Landry, 2003) while other plant extracts (*Melissa*, *Origanum* and *Salvia*) (10 ml/pig for 10 days) improved muscle colour stability when incorporated into porcine diets (Lahucky, Nuernberg, Kovac, Bucko, & Nuernberg, 2010). In the studies reported, variations in the efficacy of dietary antioxidants on the colour stability of pork meat may also be attributed to differences in factors such as storage temperature and packaging treatment. Interactions between polysaccharides and proteins have previously been reported in the scientific literature however, knowledge of the role of these interactions in relation to functionality in complex multiphasic systems is limited (Doublier, Garnier, Renard, & Sanchez, 2000). The lack of an effect of dietary L/F on the colour stability of pork (a^* redness values) indicates no interaction between polysaccharides (L/F) and proteins (oxymyoglobin) in the present study. In addition, following ingestion and absorption, polysaccharides are potentially bio-transformed into different forms.

Table 3
Effect of dietary laminarin/fucoidan (L/F) on the surface lightness ('*L**' value), redness ('*a**' value) and yellowness ('*b**' value) values of fresh *M. longissimus dorsi* (LD) stored in modified atmosphere packs (80% O_2:20% CO_2) for up to 14 days at 4 °C.

Parameter	Treatment	Storage time at 4 °C, days				
		1	4	7	11	14
Lightness	Control	58.20 ± 0.70[a]	60.74 ± 1.13[a]	61.47 ± 1.35[a]	62.15 ± 1.42[a]	63.63 ± 1.68[a]
*L**	L/F-SD	59.04 ± 1.98	60.63 ± 1.23	62.12 ± 1.42	62.06 ± 1.55	63.39 ± 1.55
	L/F-WS	58.09 ± 1.53	59.69 ± 1.42	60.63 ± 0.92	61.73 ± 1.09	63.17 ± 1.54
Redness	Control	9.00 ± 0.90[a]	8.95 ± 0.88[a]	8.65 ± 1.11[a]	7.42 ± 0.78[a]	5.96 ± 1.15[a]
*a**	L/F-SD	10.35 ± 1.06	8.88 ± 0.81	8.26 ± 1.18	7.16 ± 0.95	5.72 ± 0.81
	L/F-WS	10.06 ± 1.09	9.34 ± 0.82	8.07 ± 0.77	7.90 ± 0.63	6.44 ± 0.93
Yellowness	Control	7.35 ± 0.80[a]	8.02 ± 0.61[a]	8.02 ± 0.72[a]	8.03 ± 0.81[a]	9.36 ± 0.80[a]
*b**	L/F-SD	8.24 ± 0.87	8.35 ± 0.63	8.52 ± 0.69	8.46 ± 0.73	8.77 ± 0.85
	L/F-WS	8.21 ± 0.64	8.35 ± 0.59	7.59 ± 0.56	8.21 ± 0.61	8.40 ± 0.48

[a] Within each parameter and storage day, mean values (± standard deviation) in the same column are not significantly different, $P > 0.05$.

3.3. Lipid oxidation in fresh LD muscle

Lipid oxidation increased in all groups over the 14 day storage period (Table 4). Overall, the levels of lipid oxidation were low with mean values ranging from 0.12 to 1.70 mg MDA/kg pork. Lowest levels of oxidation were observed in LD steaks from pigs fed with the wet supplement (L/F-WS). Trends for lipid oxidation followed the order: C > LF-SD > L/F-WS. The spray-drying process during the manufacture of L/F-SD may have negatively affected and lowered the antioxidant capacity of the L/F supplement. While trends show a positive effect on limiting lipid oxidation in both the L/F supplemented diets, a statistically significant effect on levels of lipid oxidation was observed in the L/F-WS group when 75% of pigs (n = 6) were compared to the control group ($P < 0.05$). Therefore, it was concluded that antioxidant components of laminarin and fucoidan were deposited in porcine muscle (LD) following the inclusion of L/F in animal diets.

Many *in vitro* antioxidant assays (DPPH, FRAP, TEAC, NO, and ABTS radical scavenging) have demonstrated antioxidant potential of numerous seaweed polysaccharides (Ngo, Wijesekara, Vo, Van Ta, & Kim, 2011) including sulphated polysaccharides and β-glucans from a variety of seaweeds (*Turbinaria conoides*, *Laminaria japonica*, *Sargassum fulvellum*, and *Dictyota mertensii*) (Costa et al., 2010; Ngo et al., 2011; Paiva et al., 2011; Wang et al., 2010). Limited research exists linking the antioxidant activity of seaweed extracts *in vitro* with *in vivo* studies in human or animal subjects (in particular pigs).

Seaweeds have long been used as soil fertilisers and in animal feeds for many years but historically much of the information has been subjective (Fike et al., 2001). A previous study which examined the effects of a seaweed (*Ascophyllum nodosum*)-based proprietary product on tall fescue (bunch grasses), reported that grass fertilised with the seaweed product improved antioxidant activity in grasses and subsequently increased the antioxidant activity of ruminant animals grazed on the treated grasses by increasing serum vitamin A and whole-blood selenium levels (Fike et al., 2001). This finding supports the hypothesis that dietary supplementation with seaweed can, in fact, increase antioxidant activity in animals as reported in the present study where, direct supplementation of L/F had an impact on lowering levels of lipid oxidation in porcine muscle.

3.4. Lipid oxidation in porcine liver, heart, kidney and lung tissue homogenates

The uptake and distribution of dietary antioxidant compounds such as vitamin E has been measured in various porcine tissues (Morrissey et al., 1996). Due to the chemical nature and complexity of the L/F seaweed extracts utilised in the present study, the LD muscle and tissues examined were not assayed directly for laminarin and fucoidan present in the seaweed extract. In order to determine whether antioxidant compounds present in the L/F extract were distributed throughout porcine tissues, other than the LD muscle, liver, heart, kidney and lung tissue homogenates were subjected to iron ($FeSO_4$)-induced lipid oxidation. Following $FeSO_4$ addition, lipid oxidation increased in all tissue homogenates over the 24 h storage period. Trends indicate lower levels of lipid oxidation in liver tissue homogenates, as a result of dietary L/F, however due to variation results were not statistically significant (Table 5). In heart, kidney and lung tissue homogenates, significant decreases in levels of lipid oxidation were not detected.

In a previously reported dietary study, where rats were fed with plant extracts (rosemary, grape, citrus and marigold, 0.5 g/kg diet for three weeks) with bio-efficiency in the liver, it was concluded that supplementation for 3 weeks was not sufficient to significantly modify the intensity of lipid oxidation in extra-hepatic tissues (*longissimus thoracis* and heart muscles) (Gladine et al., 2007b). In a related study, dietary laminarin extracted from brown algae, fed for 25 days (5% during 4 days followed by 10% during 21 days) was found to modulate intra-hepatic immune cells in rats thus protecting the liver from damage (Neyrinck, Mouson, & Delzenne, 2007). Such findings indicate deposition of laminarin in liver tissues following oral ingestion. Similarly, Airanthi et al. (2011) reported that mice, fed with brown seaweed extracts (*Undaria pinnatifida*, *Sargassum horneri*, and *Cystoseira hakodatensis*) (0.5% and 2%) for four weeks, had significantly lower levels of lipid hydroperoxides in liver tissue homogenates compared to controls. Supplementation of rat diets with seaweed powder

Table 4
Effect of dietary laminarin/fucoidan (L/F) on lipid oxidation (TBARS) in fresh *M. longissimus dorsi* (LD) stored in modified atmosphere packs (80% O_2:20% CO_2) for up to 14 days at 4 °C.

Treatment	Storage time at 4 °C, days				
	1	4	7	11	14
Control	0.19 ± 0.04[a]	0.24 ± 0.15[a]	0.30 ± 0.12[a]	0.78 ± 0.51[a]	1.70 ± 1.02[a]
L/F-SD	0.16 ± 0.03	0.19 ± 0.05	0.28 ± 0.12	0.94 ± 0.92	1.21 ± 0.70
L/F-WS	0.13 ± 0.02	0.12 ± 0.03	0.19 ± 0.04	0.48 ± 0.16	0.91 ± 0.45

[a] Within each storage day, mean values (± standard deviation) in the same column are not significantly different, $P > 0.05$.

Table 5
Effect of dietary laminarin/fucoidan (L/F) on iron-induced lipid oxidation (TBARS) in organ (liver, heart, kidney and lung) tissue homogenates after 24 h of storage at 4 °C.

Treatment	Storage for 24 h at 4 °C			
	Liver	Heart	Kidney	Lung
Control	3.20 ± 1.43[a]	3.26 ± 0.26[a]	3.06 ± 1.01[a]	2.99 ± 0.93[a]
L/F-SD	1.99 ± 1.54	3.34 ± 0.27	2.26 ± 1.09	2.27 ± 1.82
L/F-WS	1.46 ± 1.36	3.28 ± 0.27	2.59 ± 0.52	2.96 ± 1.30

[a] Within each organ type, mean values (± standard deviation) in the same column are not significantly different, $P > 0.05$.

Table 6
Effect of dietary laminarin/fucoidan (L/F) on the microbial status (mesophilic and psychrotrophic) of fresh *M. longissimus dorsi* (LD) stored in modified atmosphere packs (80% O_2:20% CO_2) at for up to 15 days at 4 °C.

Temp	Treatment	Storage time at 4 °C, days				
		1	5	8	12	15
30 °C	Control	4.32 ± 0.20[a]	4.72 ± 0.36[a]	6.52 ± 0.48[a]	6.99 ± 0.38[a]	7.37 ± 0.05[a]
	L/F-SD	4.49 ± 0.18	4.95 ± 0.14	6.50 ± 0.57	6.95 ± 0.56	7.27 ± 0.50
	L/F-WS	4.58 ± 0.10	4.93 ± 0.36	6.32 ± 0.38	7.47 ± 0.14	7.41 ± 0.35
4 °C	Control	3.24 ± 0.48[a]	5.68 ± 0.81[a]	7.23 ± 0.19[a]	7.88 ± 0.32[a]	8.15 ± 0.25[a]
	L/F-SD	3.34 ± 0.11	5.51 ± 0.59	7.05 ± 0.21	7.99 ± 0.36	8.20 ± 0.23
	L/F-WS	3.42 ± 0.32	5.69 ± 0.37	7.29 ± 0.14	8.06 ± 0.19	7.94 ± 0.26

[a] Within each storage temperature and day, mean values (± standard deviation) in the same column are not significantly different, $P > 0.05$.

(*Eucheuma cottonii*) (5% and 10% for 35 days) increased the activity of superoxide dismutase (antioxidant enzyme) in liver tissues of hypocholesterolemic rats (Wresdiyati, Hartanta, & Astawan, 2009).

3.5. Microbiology of fresh LD muscle

The mesophilic plate counts ranged from 4.32 to 4.58 log CFU/g on day 1 and increased to a maximum of 7.41 log CFU/g on day 15 of storage (Table 6). Psychotropic plate counts ranged from 3.24 to 3.42 log CFU/g on day 1 and increased to levels ranging from 7.94 to 8.20 log CFU/g on day 15 (Table 6). Mesophilic counts obtained are similar to previously reported values for fresh pork (Houben et al., 1998). Previously reported benefits of L/F include antimicrobial activity in test systems such as growth inhibition assays and the agar plate diffusion test, however results from the present study indicate that L/F dietary supplementation did not exert antimicrobial activity in fresh pork muscle.

Several *in vitro* antimicrobial assays demonstrate antimicrobial activities of various types of seaweed including extracts from *Ceramium rubrum*, *Mastocarpus stellatus* and *L. digitata*. An extract from *L. digitata* (31 mg dry weight/ml) demonstrated strong antibacterial activity and inhibited almost all test bacteria (12 marine and 7 prominent fish pathogenic bacteria). In addition, *L. digitata* resulted in both bacteriostatic and bacteriolytic (the destruction of bacteria by lysis) modes of action (Dubber & Harder, 2008). While antimicrobial activity of seaweed extracts has been reported using *in vitro* assays, to date, the scientific literature contains no studies demonstrating antibacterial activity of seaweed extracts in food products (Gupta & Abu-Ghannam, 2011).

4. Conclusions

The quality enhancing effects of supplementing pig diets with L/F was mediated through decreased levels of lipid oxidation in LD muscle. Addition of L/F to porcine diets did not affect the surface colour of pork meat. No antimicrobial effect of dietary L/F was observed under the experimental conditions employed in the present study. Due to concerns regarding toxicity of synthetic antioxidants, L/F may prove to be a natural antioxidant alternative when incorporated into animal feed and have application in the development of novel functional meat products. In addition L/F supplementation may enhance animal health and subsequently increase fresh meat quality. Further research is necessary to examine the effects of dietary laminarin and fucoidan levels, form and duration of feeding on the quality and shelf-life of fresh pork.

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Influence of level and duration of feeding polysaccharide (laminarin and fucoidan) extracts from brown seaweed (*Laminaria digitata*) on quality indices of fresh pork

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ABSTRACT

The effect of level (450 or 900 mg laminarin (L) and fucoidan (F) /kg feed) and duration (3 or 6 wks) of feeding a seaweed (*Laminaria digitata*) extract containing L/F on the quality of pork (*longissimus thoracis et lumborum* (LTL)) stored in modified atmosphere packs and on organ lipid stability was examined. Mechanisms of L/F antioxidant activity in LTL were evaluated. Plasma total antioxidant status, LTL pH, colour, microbiology and 'eating quality' sensory analysis were unaffected by dietary L/F. 'Visual' sensory descriptors (purchasing appeal and overall visual acceptability) were enhanced ($p < 0.05$) in L/F_{450-3} LTL. Lipid oxidation was lower ($p < 0.05$) in L/F_{450-3} and L/F_{900-3} LTL and reduced in L/F_{900-6} kidney homogenates. In cooked minced pork, lipid oxidation was not reduced by dietary L/F. Saturated fatty acids were lower ($p < 0.05$) in L/F_{900-6} LTL. Results indicated L/F in pig diets for 3 weeks enhanced pork quality.

1. Introduction

The use of antioxidants in the meat industry is an effective way to minimise or prevent lipid oxidation, retard the formation of toxic oxidation products, maintain nutritional quality and prolong shelf-life (Gupta & Abu-Ghannam, 2011). The addition of antioxidants to meat and meat products can be achieved by supplementing animal diets with specific compounds for a defined duration pre-slaughter. The level and duration of dietary antioxidant compounds necessary to enhance meat quality parameters are dependent on factors such as chemical structure and purity of the antioxidant, animal species, breed and other physiological factors. Previous strategies have investigated various levels and durations of feeding antioxidant compounds such as α-tocopheryl acetate (vitamin E), β-carotene, ascorbic acid, tea catechins, chitosan and plant extracts to pigs for the determination of an optimum level and duration combination required to enhance pork meat quality (Decker, Faustman, & Lopez-Bote, 2000).

Supplementation of α-tocopheryl acetate in porcine diets has proven effective in enhancing the oxidative stability of *longissimus dorsi* muscle which increased as a function of dietary level (20 and 200 mg/kg feed) and duration (0, 35, 126 days) of supplementation, in post mortem muscle (Morrissey, Buckley, Sisk, Lynch, & Sheehy, 1996). Similarly, dietary green tea catechins (200 mg/kg) lowered levels of lipid oxidation in *longissimus thoracis et lumborum* (LTL) pork steaks (Mason et al., 2005). Lahucky, Nuernberg, Kovac, Bucko, and Nuernberg (2010) reported that dietary supplementation of oregano extract (30 and 60 ml/day) in pig diets increased lipid stability in *longissimus thoracis* muscle as a function of level, however the same dose response was not observed with dietary supplementation of Melissa (20 and 100 ml/day).

The potential of dietary antioxidants to influence other quality parameters such as the colour stability of pork meat varies considerably (Jensen, Lauridsen, & Bertelsen, 1998). Buckley, Morrissey, and Gray (1995) reported an increase in '*a**' redness values of LTL muscle from pigs fed a high level of α-tocopheryl acetate (200 mg/kg feed) compared to pigs fed a basal level of 10 mg α-tocopheryl acetate /kg of feed. Conversely, Jensen et al. (1997) reported no influence on the colour stability of LTL pork steaks when pigs were fed increasing levels of α-tocopheryl acetate (100, 200 and 700 mg/kg feed). Similarly, Mason et al. (2005) reported green tea catechins (200 mg/kg) did not enhance the colour stability of LTL pork steaks. However, supplementation with

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ascorbic acid (0, 100, 250 or 500 mg/L) in pig diets enhanced the 'a^*' redness values of LTL muscle at the highest level (500 mg/L) (Pion, van Heugten, See, Larick, & Pardue, 2004).

Due to their complex structure, seaweed polysaccharides have diverse biological properties (Thomes, Rajendran, Pasanban, & Rengasamy, 2010). Bioactive compounds identified in seaweed extracts have demonstrated a capacity to neutralise superoxide and hydroxyl radicals (Bocanegra, Bastida, Benedí, Ródenas, & Sánchez-Muniz, 2009). Brown seaweed polysaccharides including laminarin (β-glucan) and fucoidan (a sulphated polysaccharide) exhibit bioactivities such as anti-adhesive, anticoagulant, anti-inflammatory, antioxidant and anti-tumoral properties (Eluvakkal, Sivakumar, & Arunkumar, 2010). Antioxidant activity of laminarin and fucoidan depends on several structural parameters, such as the type of sugar and glycosidic branching, molecular weight and the degree and position of sulphation (Jiménez-Escrig, Gómez-Ordóñez, Tenorio, & Rupérez, 2012). Laminarin contains two types of polymeric chains, one where glucose is attached to the end of the chain (G-chain) and the other has mannitol as the terminal reducing end (M-chain) (Choi, Kim, & Lee, 2011). Fucoidan extracted from *Laminaria digitata* is reported to contain fucose and sulphates as well as xylose, mannose, glucose, galactose and uronic acid in minor amounts (Li, Lu, Wei, & Zhao, 2008). Understanding the mechanism (inhibit/scavenge reactive species, prevent/terminate free-radical generating reactions or chelate/sequester metals) through which compounds exert their antioxidant activity can help determine the mode of action/efficacy of antioxidant compounds in muscle, following digestion (Decker et al., 2000).

Consumer concerns over the safety and toxicity of synthetic antioxidants in meat products has led to increased research into the use of natural antioxidant compounds. The perceived healthiness and structure of seaweed makes it an ideal source of bioactive compounds with antioxidant activities which may replace synthetic antioxidants in meat products. Moroney, O'Grady, O'Doherty, and Kerry (2012) reported that a brown seaweed extract, containing laminarin and fucoidan, decreased lipid oxidation in fresh pork LTL. Pigs from the same study displayed improved gut health due to laminarin and fucoidan from the dietary seaweed extract (Murphy et al., 2013). However, further investigation is necessary to determine the level and duration of feeding dietary seaweed extracts containing laminarin and fucoidan in order to optimise fresh pork quality and shelf-life.

The objective of this study was to examine the effects of supplementation of porcine diets with a polysaccharide (laminarin (L) and fucoidan (F)) (L/F) based seaweed extract at two levels (450 or 900 mg/kg feed) and durations (3 or 6 wks) of feeding, on the quality, shelf-life parameters and sensory properties of fresh *longissimus thoracis et lumborum* (LTL) steaks. The influence of dietary L/F on iron-induced lipid oxidation in porcine liver, heart, kidney and lung tissue homogenates was assessed. The mechanism of action of dietary L/F on the stability of lipids in pork meat was also investigated.

2. Materials and methods

2.1. Reagents

All chemicals used were 'AnalaR' grade obtained from Sigma-Aldrich Ireland Ltd., Arklow, Co. Wicklow, Ireland, Oxoid Ltd., Basingstoke, Hampshire, England, Fisher Scientific, Dublin, Ireland and Merck KGaA, Darmstadt, Germany. The total antioxidant status (TAS) Randox-Trolox kit was obtained from Randox Laboratories Ltd., Co. Antrim, UK. A wet formulation seaweed extract (L/F), containing laminarin (L) and fucoidan (F) was manufactured by Bioatlantis, Tralee, Co. Kerry, Ireland. The extract isolated from brown seaweed (*Laminaria digitata*), harvested in Ireland, was prepared using an acid extraction technique, details of which are industrially-confidential. The composition of L/F is outlined in Table 1.

Table 1
Composition of the seaweed extract containing laminarin (L) and fucoidan (F) (L/F).

	L/F extract
% Total solids	38.9
% Ash[a]	28.1
% Protein	0.25
% Laminarin	4.0
% Fucoidan	3.2
% Mannitol	3.3
Inclusion rate (per tonne feed) (L/F_{450})	6.25 kg
Inclusion rate (per tonne feed) (L/F_{900})	12.5 kg

[a] The ash content of the seaweed extract was 15 g/kg DM Ca, 10 g/kg DM Na, 10 g/kg DM K, 10 g/kg DM S, 250 mg/kg DM iodine, 250 mg/kg DM Fe, 20 mg/kg DM Cu and 50 mg/kg DM Zn.

2.2. Animals and diets

Thirty pigs (Large White x Landrace crosses consisting of 15 males and 15 females) (average live weight ~82 kg) were randomly assigned to one of five dietary treatments ($n = 6$) and fed ad libitum a basal diet plus the L/F extract for 3 or 6 weeks pre-slaughter, following a completely randomised experimental design. The control group were fed the basal diet for the duration of the experiment. The composition and analysis of the basal diet is reported in Moroney et al. (2012). The seaweed extract in the feed formulation was added to result in total L and F concentrations of 450 or 900 mg/kg feed. Average daily feed intake, average daily gain, feed conversion ratio and details of the dietary treatments of each group are outlined in Table 2.

The animals were housed individually (1.68 m × 1.22 m slatted pens) at Lyons Research Farm, University College Dublin, Newcastle, Co. Dublin, Ireland and fed ad libitum from hopper-style feeders. Water was supplied ad libitum from individual nipple drinkers. The ambient environmental temperature within the houses was thermostatically controlled and maintained at 22 °C. Blood samples for plasma analysis were taken prior to transportation to the abattoir (Rosderra Irish Meats Group, Edenderry, Co. Offaly, Ireland). Pigs were stunned using gas and humanely slaughtered, 3 h after feeding, following animal welfare regulations. The mean slaughter weight was 115.81 kg. The liver, heart, kidney and lungs were removed from each animal, placed in laminate vacuum pack bags (composed of polyamide/polyethylene layers), vacuum packed and stored at −18 °C for one month prior to analysis. Blood samples were centrifuged (Beckman J2-21, Beckman Instruments Inc., CA, USA) at 4720 g for 20 min at 4 °C, and plasma fractions were removed and stored at −20 °C prior to analysis. Carcasses were hung at 2 °C for 24 h at Rosderra Irish Meats Group then transported at 4 °C to the School of Food and Nutritional Sciences at University College Cork, Ireland and stored at 2 °C for a further 24 h before removal of muscles for analysis.

2.3. Plasma total antioxidant status

The total antioxidant status (TAS) of porcine blood plasma was measured as described by Moroney et al. (2012) and the results were

Table 2
Average daily feed intake (ADF) (kg/day), Average daily gain (ADG) (kg/day), Feed conversion ratio (FCR), level (mg/kg feed) and duration (weeks) of dietary L/F.

	ADF[x]	ADG[y]	FCR[z]	L level	F level	Total L/F level	Duration
Control[w]	2.13	0.810	2.62	–	–	–	6
L/F_{450-3}	2.20	0.805	2.68	250	200	450	3
L/F_{450-6}	2.18	0.812	2.68	250	200	450	6
L/F_{900-3}	2.15	0.820	2.62	500	400	900	3
L/F_{900-6}	2.13	0.810	2.63	500	400	900	6

[w] Control group fed basal diet only.
[x] SEM 0.05.
[y] SEM 0.03.
[z] SEM 0.07.

expressed as mmol of trolox equivalent antioxidant capacity (TEAC)/L plasma.

2.4. Pork processing and packaging

Longissimus thoracis et lumborum (LTL) muscles were excised from each carcass, vacuum packed and stored at 2 °C for 24 h. LTL were cut into steaks (~2.5 cm in thickness, ~45 g portion), placed in low oxygen permeable (<1 cm^3/m^2/24 h at STP) polystyrene/ethylvinylalcohol (EVOH)/polyethylene (PE) trays and flushed with 80% O_2: 20% CO_2 (modified atmosphere packs, MAP) using a vacuum-sealing unit (VS 100, Gustav Müller & Co. KG, Homburg, Germany) equipped with a gas mixer (Witt-Gasetechnik GmbH & Co. KG, Witten, Germany). Trays were covered and heat-sealed using a low oxygen permeable (3 cm^3/m^2/24 h at STP) laminated barrier film with a polyolefin heat-sealable layer. LTL steaks in MAP were stored for up to 14 days under fluorescent lighting (660 lx) at 4 °C. The gas atmosphere (% O_2 and % CO_2) in the MAP was checked using a CheckMate 9900 (PBI-DanSensor, Denmark). Immediately after gas flushing, MAP contained 75.07 ± 0.43% O_2 and 25.62 ± 0.29% CO_2. The average gas composition in MAP at the end of the 14 day storage period was 73.01 ± 0.88% O_2 and 26.83 ± 0.87% CO_2.

In the cooked pork study, LTL muscles from all treatments were trimmed of visible fat and connective tissue and minced twice through a plate with 4 mm holes (Model P114L, Talsa, Valencia, Spain), formed into patties (100 g portions) using a meat former (Ministeak burger maker, O.L Smith Co. Ltd., Italy), placed on aluminium foil lined trays and cooked at 180 °C for 20 min in a fan-assisted convection oven (Zanussi Professional, Model 10 GN1/1, Conegliano, Italy) until an internal temperature of 72 °C was reached. Cooked patties were placed either in trays and either flushed with 70% N_2: 30% CO_2 (MAP) and stored for up to 14 days as described previously, or over-wrapped with oxygen permeable low-density polyethylene film and stored aerobically for up to 3 days at 4 °C (approximately 660 lx). Immediately after gas flushing, MAP contained 67.55 ± 0.93% N_2 and 32.25 ± 0.72% CO_2. The average gas composition in MAP at the end of the 14-day storage period was 67.89 ± 0.16% N_2 and 30.98 ± 0.06% CO_2. Percent nitrogen was calculated by difference of % O_2 and % CO_2 (CheckMate 9900).

2.5. Measurement of pork quality and shelf-life parameters

Pork pH, colour (CIE 'L*' lightness, 'a*' redness and 'b*' yellowness values), lipid oxidation (2-thiobarbituric acid reactive substances (TBARS)), microbiological analysis (mesophilic and psychrotrophic total viable counts), and sensory evaluation ('visual' and 'eating quality') were measured at intervals during storage as described by Moroney et al. (2012), Moroney, O'Grady, O'Doherty, and Kerry (2013). The pH, colour measurements and lipid oxidation in fresh LTL steaks and cooked minced pork patties were recorded on days 1, 4, 7, 11 and 14 of storage. In cooked minced pork patties stored aerobically, lipid oxidation was measured on days 0, 1, 2 and 3 of storage. Microbiological analysis of fresh LTL steaks was carried out on days 1, 5, 8 and 13 of storage. Sensory analysis ('visual' and 'eating quality') of fresh LTL steaks stored in 80% O_2: 20% CO_2 was performed in duplicate by 52 naive assessors on days 1 and 7 of storage. 'Visual' sensory analysis descriptors of fresh LTL steaks were pinkness, whiteness, drip, package quality, purchasing appeal, and overall acceptability. 'Eating quality' sensory analysis descriptors of cooked LTL steaks (180 °C for 20 min in a fan-assisted convection oven) were appearance, tenderness, oxidation flavour, liking of flavour and overall acceptability.

2.6. The mechanism of action of L/F on lipid stability in pork meat

2.6.1. Fatty acid analysis

Lipids were extracted from pork samples with chloroform: methanol (2:1 v/v) according to the method by Folch, Lees, and Sloane-Stanley (1957). Fatty acid methyl esters (FAMEs) were prepared by first using 10 ml 0.5 N NaOH in methanol for 10 min at 90 °C followed by 10 ml 14% BF_3 in methanol for 10 min at 90 °C (Park & Goins, 1994). FAMEs were recovered with hexane. Prior to gas chromatography (GC) analysis, samples were dried over anhydrous sodium sulphate (0.5 g) for 1 h and stored at −20 °C. FAMEs were separated using GC (Varian 3800, Varian, Walnut Creek, CA, USA) fitted with a flame ionisation detector, using a Chrompack CP Sil 88 column (Chrompack, Middleton, The Netherlands, 100 m × 0.25 mm i.d., 0.20 μm film thickness) and helium as the carrier gas. The column oven was programmed to be held initially at 80 °C for 8 min and increased 8.5 °C/min to a final column temperature of 200 °C. The injection volume used was 0.6 μl, with automatic sample injection on a SPI 1093 splitless on-column temperature programmable injector. Peaks were integrated using the Varian Star Chromatography Workstation software (version 6.0) and peaks were identified by comparison of retention times with pure FAME standards (Nu-Chek Prep, Elysian, MN, USA). The percentage of individual fatty acids was calculated according to the peak areas relative to the total area (total fatty acids were set at 100%). Results were expressed as g/100 g FAME.

2.6.2. In vitro antioxidant activity of L/F in pork meat

Pork homogenates (10% w/v) were prepared as described by Qwele et al. (2013) for measurement of in vitro antioxidant activity. Briefly, LTL steaks (10 g) were homogenised in 0.05 M phosphate buffer (90 ml), pH 7, using an Ultra Turrax T25 homogeniser. Trolox C (1000 μg/ml), EDTA (1000 μg/ml) were added to 10% pork homogenates as positive controls for the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging and the ferrous ion chelating activity (FICA) assays. A spray-dried seaweed extract containing 9.3% L and 7.8% F, comparable to the wet extract used in the animal diets, was added to 10% pork homogentates (1000 and 3000 μg/ml) as a positive seaweed polysaccharide control for both DPPH and FICA assays. Homogenates were centrifuged at 7800 *g* for 10 min at 4 °C. The supernatants obtained were used for the estimation of the DPPH free radical scavenging and FICA activities of L/F in pork meat.

2.6.2.1. Determination of DPPH radical scavenging activity. DPPH radical-scavenging activity of LTL steaks was measured as described by Qwele et al. (2013) with slight modifications. DPPH (0.2 mM, 3 ml) in methanol was added to 0.3 ml supernatant and 2.7 ml distilled water. The mixture was vortexed and left to stand at room temperature (20–22 °C) in the dark and the precipitate formed was removed by filtering through Whatman No. 1 filter paper. The assay control contained 0.3 ml buffer (0.05 M phosphate), 2.7 ml distilled water and 3 ml of DPPH solution. Absorbances were measured against a methanol blank after 20 h at 517 nm (Cary 300 Bio, UV–Vis spectrophotometer, Varian Instruments, CA, USA). Trolox C (1000 μg/ml) and L/F (1000 and 3000 μg/ml) were used as positive controls. The DPPH radical scavenging activity expressed as percent of the control was calculated as follows:

$$\%\ \text{inhibition of DPPH} = [1-(\text{absorbance of sample}/\text{absorbance of control})] \times 100.$$

2.6.2.2. Determination of ferrous ion chelating activity (FICA). The FICA of L/F in pork meat was measured using the iron–ferrozine complex method (Yen & Wu, 1999) with slight modifications. $FeCl_2$ (2 mM, 0.1 ml) in distilled water was added to 0.5 ml supernatant and 4.2 ml distilled water. The reaction was initiated with the addition of 0.2 ml of 5 mM ferrozine in distilled water, the mixture was vortexed and left to stand at room temperature (20–22 °C) for 1 h in the dark. The assay control contained 0.5 ml buffer (0.05 M phosphate), 4.2 ml distilled water, 0.1 ml of $FeCl_2$ and 0.2 ml of ferrozine solution. At 1 h the absorbance of the solution was measured at 562 nm against a water blank using a spectrophotometer (Cary 300 Bio). EDTA (1000 μg/ml) and L/F (1000

and 3000 μg/ml) were used as positive controls. The FICA was calculated as follows:

% chelating activity
= [1−(absorbance of sample/absorbance of control)] × 100.

2.7. Statistical analysis

All analyses were performed in duplicate. Mean sample values (n = 6) for each of the five treatment groups (Control, L/F_{450-3}, L/F_{450-6}, L/F_{900-3} and L/F_{900-6}) were subjected to statistical analysis. A full repeated measures two-way analysis of variance (ANOVA) was conducted to investigate the effects of dietary L/F level (450 or 900), duration (3 wk or 6 wk) and level*duration interactions. No significances were observed between level*duration interactions. Level and duration represented the 'between-subjects' factor and the effect of time was measured using the 'within-subjects' factor. Tukey's test was used to adjust for multiple comparisons between treatment means. The analysis was performed using the SPSS 18.0 for Windows (SPSS, Chicago, IL, USA) software package.

'Visual' and 'eating quality' sensory data was analysed with ANOVA-Partial Least Squares Regression (APLSR) to process the mean data accumulated from the 52 test subjects. 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. In these models assessor and session level effects were removed using level correction. The validated explained variance for the model constructed was 13.8% on day 1 and 18.0% on day 7 and the calibrated variance was 17.5% on day 2 and 21.4% on day 7. To derive significance indications for the relationships determined in the quantitative APLSR, regression coefficients were analysed by jack-knifing which is based on cross-validation and stability plots (Martens & Martens, 1999, 2001). All analyses were performed using the Unscrambler Software, version 9.8 (CAMO ASA, Trondheim, Norway).

3. Results and discussion

3.1. Plasma total antioxidant status

The TAS of porcine plasma was 1.03, 1.01, 0.91, 1.04 and 0.99 mmol TEAC/L plasma for the control, L/F_{450-3}, L/F_{450-6}, L/F_{900-3} and L/F_{900-6}, groups respectively. Results indicated that level of L/F dietary addition or duration of feeding did not influence ($p > 0.05$) the TAS of porcine plasma. Similar findings were reported by Moroney et al. (2012), where porcine plasma TAS was not affected by L/F supplementation at a level and duration similar to L/F_{900-3}. Similarly, dried *Ascophyllum nodosum* (2.5, 5.0 or 10.0 g/kg) did not affect a range of markers for plasma oxidative status, including MDA, after inclusion in pigs diets for 28 days (Michiels et al., 2011). In the present study, pigs were fed 3 h pre-slaughter and lack of an effect of L/F on porcine plasma TAS may have been due to the rapid absorption during digestion.

3.2. Colour stability and muscle pH of fresh LTL muscle

The surface lightness '*L**' and yellowness 'b*' values increased in fresh LTL muscle over the 14-day storage period. The '*a**' redness values of LTL muscle decreased as a function of storage time and was not influenced by level or duration of feeding dietary L/F (Table 3). Similar trends in the colour stability of pork meat were reported by Moroney et al. (2012). In addition, Park et al. (2005) demonstrated that supplementation of pig diets with a polysaccharide iron complex (chitosan–alginate–Fe(II) (3 ml per day)) had no effect on LTL pork steak colour parameters.

Muscle pH of fresh pork ranged from 5.61 to 5.15 over the 15-day storage period and was unaffected by level and duration of feeding dietary L/F. The pH values reported were comparable to previously reported values (5.90–5.19) for post-mortem pork muscle from pigs fed L/F (Moroney et al., 2012). The relationship between muscle pH and pork colour (L^* and a^*) has been established where pH-induced effects on muscle proteins directly correlated with changes in the pink colour of pork, as observed in pale soft exudative (PSE) and dark firm dry pork meat (DFD) (Brewer, Zhu, Bidner, Meisinger, & McKeith, 2001). In the present study, no effect was exerted by level or duration of feeding L/F on muscle pH or the colour stability of fresh pork LTL steaks.

3.3. Lipid oxidation in fresh LTL muscle and cooked minced pork

Lipid oxidation increased in meat from all dietary groups as a function of storage time (Table 4). However, the levels of lipid oxidation were generally low with mean values ranging from 0.16–0.93 mg MDA/kg pork. Trends for lipid oxidation followed the order: C > L/F_{450-6} > L/F_{900-6} > L/F_{450-3} > L/F_{900-3}. On day 11 and 14 of storage in MAP, levels of lipid oxidation in L/F_{450-3} and L/F_{900-3} were significantly ($p < 0.05$) lower compared to the control. Moroney et al. (2012), reported protection against lipid oxidation in

Table 3
Effect of dietary laminarin/fucoidan (L/F) on the surface lightness ('*L**' value), redness ('*a**' value) and yellowness ('*b**' value) values of fresh *longissimus thoracis et lumborum* (LTL) stored in modified atmosphere packs (80% O_2: 20% CO_2) for up to 14 days at 4 °C.

Treatment	Parameter	Storage time at 4 °C, days				
		1	4	7	11	14
Control	Lightness	54.99 ± 2.83[a]	55.02 ± 2.32[a]	55.74 ± 2.14[a]	56.38 ± 2.07[a]	59.01 ± 2.06[a]
L/F_{450-3}	L^*	54.42 ± 4.09	54.73 ± 5.01	57.14 ± 3.96	57.59 ± 4.40	59.11 ± 3.53
L/F_{450-6}		53.21 ± 2.47	54.23 ± 2.59	56.17 ± 2.09	57.60 ± 1.28	58.85 ± 1.60
L/F_{900-3}		52.94 ± 2.77	54.50 ± 2.41	55.62 ± 1.55	56.01 ± 0.93	56.82 ± 1.45
L/F_{900-6}		54.54 ± 2.30	56.11 ± 1.68	58.17 ± 3.3.5	56.85 ± 2.46	58.99 ± 1.80
Control	Redness	6.75 ± 1.12[a]	6.45 ± 0.41[a]	5.53 ± 0.57[a]	4.13 ± 0.72[a]	3.73 ± 1.51[a]
L/F_{450-3}	a^*	6.80 ± 0.62	6.64 ± 0.55	5.18 ± 0.53	5.14 ± 0.78	4.18 ± 1.10
L/F_{450-6}		6.19 ± 1.01	5.79 ± 0.53	5.34 ± 1.04	4.77 ± 0.72	3.49 ± 0.76
L/F_{900-3}		5.97 ± 0.74	5.20 ± 0.76	4.97 ± 0.65	3.92 ± 0.56	3.89 ± 0.43
L/F_{900-6}		6.39 ± 1.21	5.49 ± 1.39	4.79 ± 1.20	4.00 ± 0.93	3.29 ± 1.27
Control	Yellowness	7.99 ± 0.88[a]	8.09 ± 0.44[a]	7.84 ± 0.51[a]	7.42 ± 0.46[a]	8.67 ± 1.55[a]
L/F_{450-3}	b^*	8.26 ± 0.96	8.35 ± 1.11	8.34 ± 1.04	8.85 ± 1.03	9.04 ± 1.43
L/F_{450-6}		7.46 ± 0.81	7.29 ± 0.39	7.81 ± 0.57	8.29 ± 0.82	7.95 ± 1.20
L/F_{900-3}		7.26 ± 0.58	7.22 ± 0.42	7.60 ± 0.70	7.35 ± 0.35	7.73 ± 0.46
L/F_{900-6}		7.48 ± 0.75	7.51 ± 0.83	7.89 ± 1.29	7.39 ± 0.98	7.99 ± 1.06

[a] Within each parameter and storage day, mean values (±standard deviation) in the same column are not significantly different, $p > 0.05$.

Table 4
Effect of dietary laminarin/fucoidan (L/F) on lipid oxidation (TBARS) in fresh *longissimus thoracis et lumborum* (LTL) and cooked pork patties stored in modified atmosphere packs (MAP) and aerobically for up to 14 days and 3 days, respectively, at 4 °C.

Treatment	Packaging		Storage time at 4 °C, days				
			1	4	7	11	14
Control	MAP[1]	Fresh	0.16 ± 0.01[a]	0.17 ± 0.03[a]	0.20 ± 0.04[a]	0.60 ± 0.26[a]	0.93 ± 0.33[a]
L/F_{450-3}			0.16 ± 0.02[a]	0.19 ± 0.04[a]	0.17 ± 0.06[a]	0.32 ± 0.08[b]	0.40 ± 0.16[b]
L/F_{450-6}			0.16 ± 0.02[a]	0.17 ± 0.03[a]	0.17 ± 0.04[a]	0.45 ± 0.21[ab]	0.65 ± 0.51[ab]
L/F_{900-3}			0.16 ± 0.02[a]	0.16 ± 0.02[a]	0.16 ± 0.02[a]	0.31 ± 0.10[b]	0.37 ± 0.13[b]
L/F_{900-6}			0.16 ± 0.01[a]	0.16 ± 0.03[a]	0.18 ± 0.03[a]	0.35 ± 0.09[ab]	0.52 ± 0.26[ab]
Control	MAP[2]	Cooked	1.78 ± 0.42[a]	2.91 ± 0.60[a]	2.90 ± 0.55[a]	3.07 ± 0.60[a]	2.89 ± 0.45[a]
L/F_{450-3}			1.76 ± 0.49[a]	2.66 ± 0.62[a]	2.39 ± 0.48[a]	2.63 ± 0.68[a]	2.54 ± 0.57[a]
L/F_{450-6}			2.03 ± 0.36[a]	3.05 ± 1.17[a]	3.12 ± 0.71[a]	2.96 ± 0.47[a]	3.02 ± 0.59[a]
L/F_{900-3}			1.71 ± 0.18[a]	2.28 ± 0.19[a]	2.47 ± 0.29[a]	2.65 ± 0.45[a]	2.55 ± 0.36[a]
L/F_{900-6}			2.11 ± 0.50[a]	3.04 ± 0.94[a]	3.08 ± 0.57[a]	3.35 ± 0.81[a]	2.65 ± 0.48[a]
			0	1	2	3	
Control	Aerobic	Cooked	1.21 ± 0.34[a]	2.03 ± 0.54[a]	2.59 ± 0.53[a]	3.39 ± 0.88[a]	
L/F_{450-3}			1.26 ± 0.18[a]	1.85 ± 0.58[a]	2.32 ± 0.77[a]	2.94 ± 1.02[a]	
L/F_{450-6}			1.08 ± 0.29[a]	2.34 ± 0.51[a]	3.13 ± 0.74[a]	3.75 ± 0.76[a]	
L/F_{900-3}			1.09 ± 0.14[a]	1.81 ± 0.22[a]	2.40 ± 0.42[a]	3.11 ± 0.54[a]	
L/F_{900-6}			1.40 ± 0.19[a]	2.51 ± 0.54[a]	2.92 ± 0.34[a]	3.48 ± 0.35[a]	

[1](80% O_2: 20% CO_2), [2](70% N_2: 30% CO_2).
[ab]Within each parameter and storage day, mean values (±standard deviation) in the same column bearing different superscripts are significantly different, $p < 0.05$.

LTL steaks potentially due to deposition of antioxidant components in L/F in pork muscle following dietary supplementation at a level similar to L/F_{900-3}.

Recent studies have shown that dietary L/F modulated gastrointestinal physiology, improved digestion and increased absorptive functions of the pig intestine (Heim et al., 2014). The degree of digestibility of the seaweed polysaccharides increased with feeding duration, which subsequently increased nutrient digestibility (O'Doherty, Dillon, Figat, Callan, & Sweeney, 2010). In the present study, L/F_{450-3} and L/F_{900-3} was sufficient to increase lipid stability in LTL steaks. Uptake and increased utilisation of the polysaccharides by gut microbes may be responsible for the variation observed in lipid stability of L/F_{450-6} and L/F_{900-6} compared to feeding L/F for 3 weeks.

In cooked pork patties stored aerobically and in MAP, lipid oxidation increased in all treatments over the 14-day storage period (Table 4). Lowest levels of lipid oxidation for cooked pork patties stored aerobically and in MAP were observed in L/F_{450-3} and L/F_{900-3} and results were not significantly different from the controls. Results indicated low to moderate thermal stability of L/F in cooked pork meat however the behaviour of dietary seaweed polysaccharides during cooking requires further investigation. Lipid oxidation levels were greater than those observed for raw LTL muscle due to the pro-oxidative nature of the mincing and cooking processes. In cooked patties stored in MAP, lipid oxidation was lower than in patties stored aerobically due to the low oxygen levels present in MAP. In the present study, although not statistically significant, trends showed that dietary L/F supplementation for 3 weeks resulted in slightly lower levels of lipid oxidation compared to the controls for cooked patties stored in both packaging conditions.

3.4. Lipid oxidation in porcine liver, heart, kidney and lung tissue homogenates

Liver, heart, kidney and lung tissue homogenates were subjected to iron ($FeSO_4$)-induced lipid oxidation to estimate the distribution of L/F extract in porcine organ tissues. Following $FeSO_4$ addition, lipid oxidation increased in all tissue homogenates over the 24 h storage period. While not significantly different, trends indicated lower levels of lipid oxidation in kidney and lung tissue homogenates, as a result of dietary L/F (Table 5). Moroney et al. (2012) reported slightly lower levels of lipid oxidation in porcine liver tissue homogenates after dietary supplementation of a spray-dried seaweed extract similar to L/F_{900-3}. In the present study, similar decreases in levels of lipid oxidation of liver tissues after L/F supplementation were not detected. Lynch, Sweeney, Callan, O'Sullivan, and O'Doherty (2010) suggested that pig maturity influenced the digestion, breakdown and subsequent availability of fermentable polysaccharides entering the large intestine. Pigs used in the present study (115.81 kg) were larger in size than pigs (29.7 kg) investigated by Moroney et al. (2012). The differences in lipid stability of organ tissues observed between the two studies may be attributed to maturity of the pigs at the time of slaughter.

Several studies have shown that dietary seaweed extracts, containing laminarin and fucoidan individually, can accumulate and positively influence stress markers in animal liver, heart, kidney and lung tissues (Thomes et al., 2010). In rat lung tissue, deposition of antioxidant components from dietary laminarin (200 or 400 mg/kg body weight) was found to significantly reduce malondialdehyde (MDA) levels (Cheng, Liang, Li, & Jin, 2011). In the present study, the maximum level and duration (L/F_{900-6}) was the most effective (not significantly) of all treatments examined at reducing iron-induced oxidation of porcine kidney and lung tissue homogenates compared to the control. The digestion of soluble dietary fibre has been linked to changes in microbiota which consequently influences absorption rate of minerals and other nutrients (Scholz-Ahrens, Schaafsma, van den Heuvel, & Schrezenmeir, 2001). The digestion and utilisation of dietary L/F by the porcine gut to support immunological health may have influenced the bioavailability and subsequent deposition of L/F and minerals from the extract in the organ tissues. Increased availability of iron and copper, known initiators of lipid oxidation, from the seaweed extract may have counterbalanced the antioxidant potential of dietary L/F.

Table 5
Effect of dietary laminarin/fucoidan (L/F) on iron-induced lipid oxidation (TBARS) in organ (liver, heart, kidney and lung) tissue homogenates after 24 h storage at 4 °C.

Treatment	Storage for 24 h at 4 °C			
	Liver	Heart	Kidney	Lung
Control	2.91 ± 0.76[a]	1.42 ± 0.88[a]	2.68 ± 0.82[a]	1.26 ± 0.55[a]
L/F_{450-3}	2.93 ± 0.81	1.21 ± 0.70	2.52 ± 0.46	1.21 ± 0.71
L/F_{450-6}	3.21 ± 0.85	1.76 ± 0.81	2.72 ± 0.55	1.55 ± 0.77
L/F_{900-3}	3.10 ± 0.67	1.52 ± 0.31	1.72 ± 0.90	1.20 ± 0.65
L/F_{900-6}	2.98 ± 0.39	1.17 ± 0.35	1.59 ± 0.65	0.86 ± 0.14

[a] Within each organ type, mean values (±standard deviation) in the same column are not significantly different, $p > 0.05$.

3.5. Microbiology of fresh LTL muscle

The mesophilic plate counts of fresh LTL muscle ranged from ~2.7 to ~3.2 log cfu/g on day 1 and increased to a maximum of ~7.0 log cfu/g on day 13 of storage (Table 6). Psychrotrophic plate counts ranged from ~4.0 to ~5.0 log cfu/g on day 1 and increased to levels ranging from ~9.9 to ~10.5 log cfu/g on day 13 (Table 6). Mesophilic and psychrotrophic counts are in agreement with previously reported values from Moroney et al. (2012). Results from the present study indicated dietary L/F, regardless of level or duration of feeding, did not exert antimicrobial activity in fresh LTL steaks.

The biological activity of sulphated polysaccharides can vary between species and has been linked to the molecular weight and sulphated content as well as the position of sulphate groups (Li et al., 2008). Antimicrobial properties of seaweed extracts are influenced by extraction solvents, form and concentration of compounds present, and activity has been reported in extracts prepared from a range of seaweed species (Bansemir, Blume, Schröder, & Lindequist, 2006; Cox, Hamilton Turley, Rajauria, Abu-Ghannam, & Jaiswal, 2013). Crude extracts containing sulphated polysaccharides from *Gracilaria ornata* exhibited no antimicrobial activity against seven bacteria (*B. subtilis*, *S. aureus*, *E. aerogens*, *E. coli*, *P. aeruginosa*, *S. choleraesuis* and *S. typhi*) (plate diffusion method). Amorim et al. (2012) postulated the absence of antimicrobial activity of sulphated polysaccharides may be due to the inability to interact with the cell wall of bacteria as a result of charge and the repulsion of the sulphated groups. In the present study, the lack of antimicrobial activity in the LTL steaks may be attributed to the form and concentration of compounds deposited in the muscle.

3.6. Sensory evaluation of fresh and cooked LTL muscle

In 'visual' sensory analysis of LTL steaks (Table 7), no significant trends were observed for 'pinkness' or 'whiteness' with the exception of L/F_{450-3} which was significantly ($p < 0.05$) positively correlated to appearing more (pink) on day 1. Instrumental '*a**' redness values support such findings indicating that colour was unaffected by level and duration of feeding dietary L/F. On day 1, L/F_{900-3} was significantly ($p < 0.001$) positively correlated to having a high drip loss while L/F_{900-6} was significantly ($p < 0.01$) negatively correlated to having no drip loss. On day 7, the control was significantly ($p < 0.001$) positively correlated to having a high drip loss while L/F_{450-3} was significantly ($p < 0.001$) negatively correlated to having no drip loss. Although not significant, L/F_{450-6}, L/F_{900-3}, L/F_{900-6} were also negatively correlated to having no drip loss on day 7, which may indicate that deposition of components of the L/F extract led to an increase in stability in the protein matrix leading to reduced drip during storage. On day 7, L/F_{450-3} was significantly ($p < 0.05$) positively correlated to purchasing appeal and overall visual acceptability while the control was significantly ($p < 0.05$) negatively correlated to purchasing appeal and overall visual acceptability. Therefore, the 'visual' sensory properties of L/F_{450-3} pork patties were enhanced by dietary L/F supplementation according to the sensory panellists.

No significant trends were observed for 'eating quality' sensory analysis including appearance, texture, and overall acceptability of the cooked LTL muscle (Table 7). In a previously reported study, the addition of dried *Himanthalia elongata* (5%) to low-fat frankfurters resulted in less acceptable products, due mainly to the flavour of the seaweed (López-López, Cofrades, Yakan, Solas, & Jiménez-Colmenero, 2010). In the present study, 'eating quality' sensory analysis was unaffected by level and duration of feeding of dietary L/F. Dietary supplementation where the L/F extract is subjected to porcine digestive and metabolic processes, may serve to eliminate possible negative organoleptic detection of flavourants by consumers. This indicates that seaweed extracts may be incorporated into porcine tissues via the animal's diet without damaging consumer preferences for pork meat.

3.7. Determination of L/F mechanism of action on lipid stability in pork muscle

The mechanism of antioxidant activity of L/F in pork meat may be attributed to one or a number of chemical reactions or pathways such as immunomodulatory activity in the gut, influence on the fatty acid composition as well as antioxidant activity (radical scavenging and ferrous ion chelating) in the muscle.

3.7.1. Improvement of porcine gut health

When used as feed additives in pig diets, components of the L/F extract have the potential to enhance porcine gut health by improving the immune system and altering the intestinal microbiota before being absorbed and deposited in the muscle (Gahan, Lynch, Callan, O'Sullivan, & O'Doherty, 2009). The immune function is specially linked to the release of reactive oxygen species (ROS), the excess of which must be eliminated by endogenous antioxidant defences. ROS which are not counteracted by the antioxidant defences of the cell can become a source of damage to DNA, lipids and proteins in the animal. Molecular damage resulting from oxidative stress can lead to significant damage to cell structures and even moderate oxidation can trigger cell death (De la Fuente, 2002). Immunomodulatory activities of L/F, have been reported, in mammals through modification of macrophage activity resulting in increased immune function (Castro, Zarra, & Lamas, 2004; Li et al., 2008). It is postulated that the lipid antioxidant activity of L/F in pork meat may be mediated through enhanced immune function as a result of dietary polysaccharide supplementation.

3.7.2. Changes in fatty acid composition of pork muscle

Lynch et al. (2010) reported that dietary supplementation of L/F influenced short chain fatty acid (SCFA) production in pigs. Polysaccharides such as L/F undergo fermentation in the cecum and large

Table 6
Effect of dietary laminarin/fucoidan (L/F) on the microbial status (mesophilic and psychrotrophic TVC) of fresh *longissimus thoracis et lumborum* (LTL) stored in modified atmosphere packs (80% O_2: 20% CO_2) at for up to 13 days at 4 °C.

Treatment			Storage time at 4 °C, days			
	Temp		1	5	8	13
Control	30 °C	Mesophilic	2.84 ± 0.27[a]	3.78 ± 0.01[a]	4.83 ± 0.22[a]	6.58 ± 0.39[a]
L/F_{450-3}			2.77 ± 0.20	4.03 ± 0.05	5.14 ± 0.49	5.46 ± 0.92
L/F_{450-6}			3.16 ± 0.08	3.76 ± 0.19	6.14 ± 0.73	6.83 ± 0.84
L/F_{900-3}			2.66 ± 0.26	3.78 ± 0.02	5.20 ± 0.41	7.01 ± 0.53
L/F_{900-6}			2.70 ± 0.30	3.65 ± 0.25	4.73 ± 0.55	6.49 ± 0.55
Control	4 °C	Psychrotrophic	4.42 ± 0.40[a]	4.80 ± 0.43[a]	6.49 ± 0.83[a]	9.90 ± 0.73[a]
L/F_{450-3}			4.68 ± 0.77	4.92 ± 0.74	6.54 ± 0.31	10.17 ± 0.20
L/F_{450-6}			4.95 ± 0.28	5.69 ± 0.29	7.24 ± 0.43	10.50 ± 0.07
L/F_{900-3}			4.00 ± 0.42	5.18 ± 0.21	6.37 ± 0.37	10.49 ± 0.02
L/F_{900-6}			4.12 ± 0.18	4.65 ± 0.30	6.77 ± 0.09	10.29 ± 0.04

[a] Within each storage temperature and day, mean values (±standard deviation) in the same column are not significantly different, $p > 0.05$.

Table 7
Significance of regression coefficients (ANOVA values) for fresh *longissimus thoracis et lumborum* (LTL) as derived by jack-knife uncertainty testing.

Parameter	Storage time, day/treatment									
	Control		L/F_{450-3}		L/F_{450-6}		L/F_{900-3}		L/F_{900-6}	
	1	7	1	7	1	7	1	7	1	7
Visual sensory analysis										
Pinkness	0.61	−0.40	0.01*	0.45	0.80	0.93	−0.31	0.48	−0.47	0.55
Whiteness	−0.64	−0.99	−0.07	0.99	−0.81	0.99	0.39	0.99	0.52	0.99
Drip	0.27	0.00***	0.23	−0.00***	0.72	−0.93	0.00***	−0.23	−0.01**	−0.57
Packaging quality	−0.67	0.99	−0.66	−0.99	−0.78	−0.99	0.65	−0.99	0.64	−0.99
Purchasing appeal	−0.69	−0.03*	−0.79	0.05*	−0.56	0.93	0.75	0.29	0.73	0.56
Overall acceptability	−0.41	−0.03*	−0.68	0.05*	−0.51	0.93	0.59	0.27	0.51	0.56
Eating quality sensory analysis										
Appearance	0.81	−0.54	0.70	0.55	0.87	0.93	−0.75	0.61	−0.78	0.56
Tenderness	−0.09	0.55	−0.56	−0.55	−0.63	−0.93	0.35	−0.61	0.20	−0.72
Oxidation flavour	−0.41	−0.93	−0.31	0.93	−0.74	0.95	0.18	0.92	0.23	0.93
Liking of flavour		0.83	−0.94	0.74	0.94	0.88	0.96	−0.78	0.94	−0.80
Overall acceptability	0.81	0.50	0.68	−0.52	0.87	−0.93	−0.75	−0.50	−0.77	−0.71

* 95% significance, $p < 0.05$.
** 99% significance, $p < 0.01$.
*** 99.9% significance, $p < 0.001$.

intestine by the anaerobic cecal and colonic microbiota resulting in multiple groups of metabolites of which, SCFA are the major group. The most abundant SCFA are acetate, propionate and butyrate (den Besten et al., 2013). SCFA can be transported from the intestinal lumen into the blood and taken up by organs where they act as substrates or signal molecules (Wong, de Souza, Kendall, Emam, & Jenkins, 2006). SCFA, in particular acetate, have been linked to the synthesis of cholesterol and long chain fatty acids as well as being a substrate for glutamine and glutamate synthesis (den Besten et al., 2013). Therefore the potential exists for dietary L/F to influence the FA profile of pork meat.

The fatty acid composition of pork meat for all dietary treatments is presented in Table 8. Susceptibility to lipid oxidation in pork meat is largely determined by the level of unsaturated fatty acids and pro-oxidants present in the muscle (Decker et al., 2000). The fatty acid composition of the control is comparable to previously reported values for fresh pork meat (Wood et al., 2004). L/F_{900-3} was lower ($p < 0.05$) in stearic acid; L/F_{900-6} was lower ($p < 0.05$) in stearic and arachidic acid relative to the control, resulting in a decreased ($p < 0.05$) total level of saturated ($\sum$ SFA) fatty acids in L/F_{900-6}. Trends indicated that all pigs fed dietary L/F had lower levels of saturated ($\sum$ SFA) fatty acids. In pork meat, stearic acid content has been linked to fat hardness due to the saturated nature of this fatty acid. However, in the present study, the tenderness sensory descriptor of cooked LTL muscle was unaffected by stearic acid level. Trends also suggested that higher levels of polyunsaturated ($\sum$ PUFA) fatty acids and higher polyunsaturated:saturated (P:S) ratios in meat from all dietary seaweed treatments relative to the control. L/F_{900-6} displayed enhanced lipid stability in addition to significantly lower ($p < 0.05$) saturated fatty acids suggesting dietary

Table 8
Effect of dietary laminarin/fucoidan (L/F) on fatty acid composition in fresh *longissimus thoracis et lumborum* (LTL).

	Treatment				
	Control	L/F_{450-3}	L/F_{450-6}	L/F_{900-3}	L/F_{900-6}
Lauric C12:0	0.12 ± 0.08[a]	0.12 ± 0.05[a]	0.13 ± 0.05[a]	0.11 ± 0.03[a]	0.12 ± 0.06[a]
Myristic C14:0	0.83 ± 0.20[a]	0.84 ± 0.30[a]	0.85 ± 0.18[a]	0.87 ± 0.13[a]	0.89 ± 0.13[a]
Palmitic C16:0	22.18 ± 0.73[a]	21.95 ± 1.51[a]	22.09 ± 0.63[a]	21.91 ± 0.47[a]	21.56 ± 0.97[a]
Stearic C18:0	13.84 ± 1.29[a]	12.97 ± 0.76[ac]	13.21 ± 0.38[ad]	12.59 ± 0.40[bcd]	11.65 ± 0.45[b]
Arachidic C20:0	0.26 ± 0.05[a]	0.22 ± 0.04[ab]	0.23 ± 0.03[ab]	0.22 ± 0.01[ab]	0.17 ± 0.09[b]
ΣSFA	37.24 ± 2.08[a]	36.10 ± 1.65[ab]	36.51 ± 1.02[ab]	35.69 ± 0.73[ab]	34.39 ± 1.25[b]
t-Palmitoleic C16:1 *trans*	0.28 ± 0.02[a]	0.28 ± 0.06[a]	0.29 ± 0.04[a]	0.30 ± 0.03[a]	0.27 ± 0.04[a]
Palmitoleic C16:1 *cis*	1.71 ± 0.43[a]	1.80 ± 0.73[a]	1.83 ± 0.27[a]	2.04 ± 0.44[a]	2.25 ± 0.42[a]
Elaidic C18:1 *trans*	0.37 ± 0.10[a]	0.37 ± 0.18[a]	0.38 ± 0.13[a]	0.38 ± 0.10[a]	0.30 ± 0.09[a]
Oleic C18:1 ($n-9$)	31.11 ± 5.35[a]	27.35 ± 6.63[a]	28.92 ± 3.54[a]	29.36 ± 3.76[a]	32.40 ± 4.86[a]
Vaccenic C18:1 ($n-7$)	2.98 ± 0.66[a]	3.00 ± 0.55[a]	3.50 ± 0.17[a]	3.29 ± 0.25[a]	3.58 ± 0.22[a]
ΣMUFA	36.46 ± 5.76[a]	32.77 ± 7.48[a]	34.92 ± 3.68[a]	35.37 ± 3.90[a]	38.81 ± 5.19[a]
Linoleic C18:2 ($n-6$)	14.85 ± 3.85[a]	17.73 ± 4.00[a]	15.67 ± 2.14[a]	16.43 ± 2.41[a]	16.28 ± 3.61[a]
γ-Linolenic C18:3 ($n-6$)	0.05 ± 0.04[a]	0.11 ± 0.04[a]	0.09 ± 0.05[a]	0.07 ± 0.04[a]	0.06 ± 0.03[a]
α-Linolenic C18:3 ($n-3$)	0.77 ± 0.16[a]	0.86 ± 0.11[a]	0.83 ± 0.19[a]	0.92 ± 0.20[a]	0.86 ± 0.15[a]
Dihomo-γ-linolenic C20:3 ($n-6$)	0.25 ± 0.06[a]	0.35 ± 0.13[a]	0.32 ± 0.07[a]	0.28 ± 0.07[a]	0.28 ± 0.07[a]
Arachidonic C20:4 ($n-6$)	2.56 ± 1.11[a]	3.39 ± 1.56[a]	3.16 ± 1.02[a]	2.85 ± 0.88[a]	2.67 ± 1.02[a]
Eicosapentaenoic C20:5 ($n-3$)	0.17 ± 0.13[a]	0.19 ± 0.10[a]	0.18 ± 0.06[a]	0.17 ± 0.06[a]	0.13 ± 0.09[a]
Docosatetraenoic C22:4 ($n-6$)	0.34 ± 0.15[a]	0.46 ± 0.23[a]	0.41 ± 0.10[a]	0.40 ± 0.16[a]	0.35 ± 0.12[a]
Docosapentaenoic C22:5 ($n-3$)	0.39 ± 0.17[a]	0.57 ± 0.26[a]	0.54 ± 0.17[a]	0.47 ± 0.15[a]	0.45 ± 0.17[a]
Docosahexaenoic C22:6 ($n-3$)	0.07 ± 0.03[a]	0.12 ± 0.07[a]	0.07 ± 0.09[a]	0.08 ± 0.05[a]	0.08 ± 0.06[a]
ΣPUFA	19.44 ± 5.29[a]	23.77 ± 6.19[a]	21.28 ± 3.42[a]	21.67 ± 3.31[a]	21.17 ± 4.93[a]
P:S ratio[#]	0.43 ± 0.13[a]	0.52 ± 0.13[a]	0.46 ± 0.07[a]	0.49 ± 0.07[a]	0.50 ± 0.12[a]
18:2$n-6$:18:3$n-3$	19.89 ± 6.49[a]	21.36 ± 7.26[a]	20.12 ± 7.57[a]	18.56 ± 4.53[a]	19.24 ± 4.47[a]
Σ$n-6$:Σ$n-3$	13.48 ± 1.31[a]	13.49 ± 1.63[a]	12.64 ± 1.68[a]	12.86 ± 1.43[a]	13.52 ± 1.69[a]

[#]P:S ratio = (C18:2$n-6$ + C18:3$n-3$)/(C12:0 + C14:0 + C16:0 + C18:0).
[ab]Within each fatty acid, mean values (±standard deviation) in the same row bearing different superscripts are significantly different, $p < 0.05$.

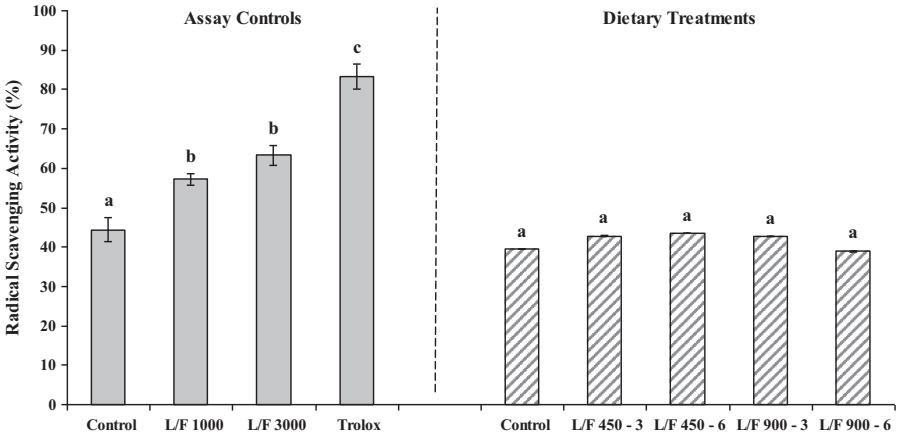

Fig. 1. Effect of dietary laminarin/fucoidan (L/F) on DPPH free radical scavenging in fresh *longissimus thoracis et lumborum* (LTL) muscle. [abc]Within each dataset (assay controls/dietary treatment), mean values (±standard deviation error bars) bearing different superscripts are significantly different, $p < 0.05$. (▩), Direct addition to meat homogenates; (▨), Dietary treatments.

addition of L/F beneficially influenced the fatty acid profile of LTL muscle without detrimental effects on lipid stability. This shift in the ratio of P:S may benefit consumers seeking meat with reduced saturated fat levels.

3.7.3. Free radical scavenging activity of L/F extract in pork muscle

The DPPH free radical scavenging activity of laminarin, fucoidan and a variety of brown seaweeds (i.e. *Fucus vesiculosus*, *Laminaria japonica*, *Sargassum plagiophyllum*, *Sargassum vulgare*) has been reported previously (Choi et al., 2011; Dore et al., 2013; Suresh et al., 2012; Vo & Kim, 2013). Free radical scavenging activity and protection against ROS is a potential mechanism of action of dietary L/F deposited in the muscle tissue. The free radical scavenging activity of fresh pork from the dietary treatments (L/F_{450-3}; L/F_{450-6}; L/F_{900-3}; L/F_{900-6}) ranged from 38.92 to 43.45% and was similar to the control (39.49%) (Fig. 1). A spray-dried seaweed extract containing L/F and Trolox were directly added to pork meat (positive controls) to determine in vitro radical scavenging activity. Direct addition of L/F (1000 and 3000 μg/ml) and Trolox (1000 μg/ml) to pork meat resulted 57.2%, 63.3% and 83.2% free radical scavenging activity, respectively. The antioxidant activity of fucoidan has been related to the sulphate content, position, molecular weight and sugar composition (Sinurat & Marraskuranto, 2013). In the present study, the seaweed extract exhibited free-radical scavenging activity in vitro, however, scavenging activity was not detected in pork meat following dietary supplementation with L/F. The lack of free-radical scavenging activity is potentially due to biotransformation of L/F during ingestion and absorption by the porcine gut before deposition in the muscle resulting in compounds un-reactive with the DPPH radical.

3.7.4. Ferrous ion chelating activity of L/F extract in pork muscle

Transition metals present in muscle foods such as iron are known to initiate and accelerate lipid oxidation in food systems. Low to moderate ferrous ion chelating activities of brown seaweeds (*Sargassum filipendula* and *Laminaria japonica*) have been previously reported, and activity was attributed to the presence of sulphated polysaccharides (Costa et al., 2011; Wang, Zhang, Zhang, & Li, 2008). Analysis of the L/F extract (rich in sulphated polysaccharides) (1000 and 3000 μg/ml) utilised in the present study, indicated no FICA whereas EDTA (positive control) resulted in 90.95% FICA. Iron chelating activity is dependent on the complex structural characteristics of seaweed polysaccharides (Mak, Hamid, Liu, Lu, & White, 2013). The L/F extract may undergo structural modification (biotransformation) during porcine digestion and potentially exhibit antioxidant activity mediated through ferrous ion chelating activity in pork meat. FICA of pork meat fractions from each dietary treatment ranged from 17.65–25.10% (similar to the control) (Fig. 2). Results indicated that dietary L/F did not influence FICA in LTL muscle and antioxidant activity of L/F is most likely due to a combination of the proposed mechanisms discussed.

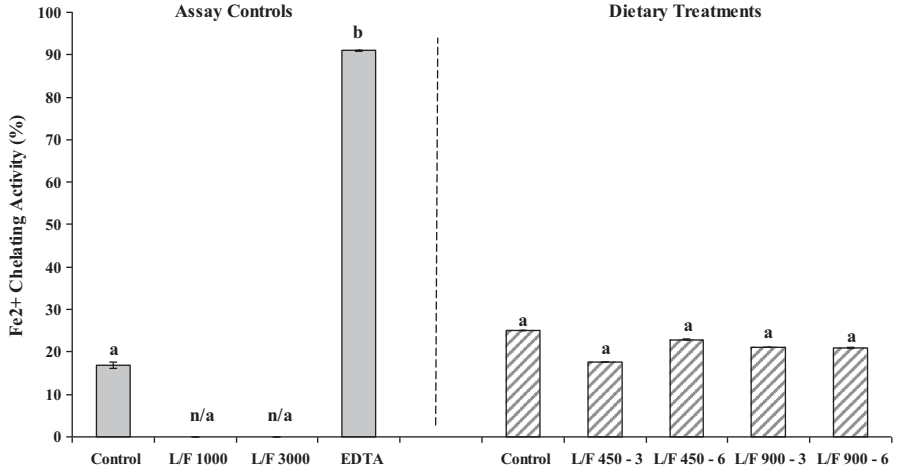

Fig. 2. Effect of dietary laminarin/fucoidan (L/F) on ferrous ion chelating activity (FICA) in fresh *longissimus thoracis et lumborum* (LTL) muscle. [ab]Within each dataset (assay controls/dietary treatment), mean values (±standard deviation error bars) bearing different superscripts are significantly different, $p < 0.05$. (▩), Direct addition to meat homogenates; (▨), Dietary treatments. [n/a] No activity.

4. Conclusions

Dietary supplementation of L/F at 3 weeks, irrespective of level, resulted in enhanced pork meat due to deposition of marine-derived bioactive antioxidant components in LTL muscle. Antioxidant response factor, dependant on duration of supplementation, was most likely attributed to a number of mechanisms. Dietary L/F reduced saturated fatty acids and lowered lipid oxidation in LTL muscle. Due to complexity of the extract and porcine metabolic pathways, it is unclear if free radical scavenging abilities of the extract were responsible for the antioxidant activity observed in LTL muscle. Meat quality enhancing effects of seaweed polysaccharides may be mediated through health promoting effects of gut-associated immunity. The improved fatty acid profile with enhanced lipid stability of pork meat without impact on tenderness, flavour or other sensory properties suggests dietary supplementation of seaweed extracts containing laminarin and fucoidan could result in an enhanced pork meat product.

Conflict of interest

The authors wish to confirm that there are no known conflicts of interest associated with this publication.

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Effect of a brown seaweed (*Laminaria digitata*) extract containing laminarin and fucoidan on the quality and shelf-life of fresh and cooked minced pork patties

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ABSTRACT

A spray-dried seaweed extract containing laminarin (L, 9.3%) and fucoidan (F, 7.8%) (L/F extract) from brown seaweed (*Laminaria digitata*) was added directly to minced pork (*M. longissimus dorsi*) (LD) at levels of 0.01%, 0.1% and 0.5% (w/w). Fresh and cooked minced pork patties were stored in modified atmosphere packs containing 80% O_2:20% CO_2 and 70% N_2:30% CO_2, respectively, for up to 14 days at 4 °C. The L/F extract reduced the surface redness ('*a**' values) of fresh patties as a function of concentration. The L/F extract (0.5%) exerted the greatest lipid pro-oxidant activity in fresh patties. The L/F extract (0.5%) significantly decreased ($P < 0.05$) lipid oxidation in cooked patties. The L/F extract had no effect on the microbiological status, pH, water holding capacity (WHC) or cook loss of patties. Pork patties containing 0.01% L/F were preferred by sensory panellists. Further research will focus on the use of refined purified seaweed extracts in functional meat products.

1. Introduction

The use of seaweed (macroalgae) or seaweed extracts as food additives is growing in popularity due to the vast range of functional properties they impart in food products. Seaweeds contain high proportions of polysaccharides, proteins, minerals, and vitamins and have low lipid content (Gómez-Ordóñez, Jiménez-Escrig, & Rupérez, 2010). Seaweed polysaccharides are a potential source of soluble and insoluble dietary fibre. Soluble seaweed polysaccharides exhibit higher water holding capacity than cellulosic (insoluble) fibres. Soluble dietary fibres demonstrate the ability to increase viscosity, form gels and/or act as emulsifiers and are also characterised by a capacity to reduce both glycemic response and plasma cholesterol in humans (Elleuch et al., 2011; Venugopal, 2008).

The cell walls of brown seaweed (*Phaeophyta*) contain polysaccharide compounds such as laminarin and fucoidan (soluble fibres). Laminarin, a β-polymer of glucose, is the main storage polysaccharide in algae. Fucoidan, a sulphated heteropolysaccharide, is composed primarily of L-fucose and protects seaweed from desiccation (Anastasakis, Ross, & Jones, 2011; Bocanegra, Bastida, Benedí, Ródenas, & Sánchez-Muniz, 2009). Seaweed polysaccharides, including laminarin and fucoidan are reported to possess antioxidant (Choi, Kim, & Lee, 2011), anti-tumour, anticoagulant, antiviral, and antibacterial activities (Costa et al., 2010; Wang et al., 2007).

The development of functional meats with enhanced physiochemical and health-promoting properties may be achieved by adding seaweeds or seaweed extracts containing bioactive components into meat and meat products. Bioactive compounds may be incorporated by supplementation of animal diets or by direct addition during meat processing (Khan et al., 2011). In a recent study carried out by our research group, supplementation of pig diets with brown seaweed (*Laminaria digitata*) extracts containing laminarin and fucoidan enhanced the oxidative stability of fresh pork meat (Moroney, O'Grady, O'Doherty, & Kerry, 2012). Similarly, Diaz-Rubio, Serrano, Borderias, and Saura-Calixto (2011) reported that the direct addition of dietary fibre from *Fucus vesiculosus* inhibited lipid oxidation in fish mince muscle.

Recent scientific studies have also examined the functionality of macroalgae (powdered/dried *Himanthalia elongata*, *Undaria pinnatifida*, *Porphyra umbilicalis*, and *Laminaria japonica*) in processed meat products, such as beef or pork burgers/patties, frankfurters, restructured poultry steaks, and pork meat emulsion systems (Choi et al., 2012; Cofrades, López-López, Ruiz-Capillas, Triki, & Jiménez-Colmenero, 2011; López-López, Cofrades, & Jiménez-Colmenero, 2009; López-López, Cofrades, Yakan, Solas, & Jiménez-Colmenero, 2010). Pigments present in seaweed can influence meat product colour depending on the seaweed/extract type and concentration added (Choi et al., 2012; Jiménez-Colmenero et al., 2010; López-López, Cofrades, Ruiz-Capillas, & Jiménez-Colmenero, 2009). Acidic seaweed components such as fucoidan and alginic acid decreased the pH of meat products containing powdered seaweeds (Choi et al., 2012; Cofrades, López-López, Solas, Bravo, & Jiménez-Colmenero, 2008). The antioxidant capacity (FRAP) of pork meat emulsion systems increased due to the high phenolic

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content of powdered seaweed added (López-López et al., 2009). Algal oils have been used to enhance the lipid profile of frankfurters (López-López, Cofrades, Ruiz-Capillas, & Jiménez-Colmenero, 2009). Soluble and insoluble dietary fibres from macroalgae increased cooking yields, improve texture, fat/water binding and emulsion stability and decreased costs in meat product formulations (Fernández-Martín, López-López, Cofrades, & Colmenero, 2009; Jiménez-Colmenero et al., 2010). Seaweeds contain a high concentration of minerals and therefore may also have potential for use as salt replacers in processed meat products (Cofrades et al., 2011).

Studies on the addition of seaweed polysaccharides to meat products are limited and merit investigation. The objective of this study was to examine the effect of the direct addition of a spray-dried seaweed (*L. digitata*) extract containing laminarin and fucoidan (L/F) on the quality and shelf-life of minced pork (*M. longissimus dorsi* (LD)) stored in modified atmosphere packs. Quality parameters examined include colour, lipid oxidation (fresh and cooked), microbiology, pH, water holding capacity (WHC), texture profile analysis (TPA), cook loss, and sensory properties during refrigerated storage at 4 °C.

2. Materials and methods

2.1. Reagents

All chemicals used were 'AnalaR' grade obtained from Sigma-Aldrich Ireland Ltd., Arklow, Co. Wicklow, Ireland, Oxoid Ltd., Basingstoke, Hampshire, England and Merck KGaA, Darmstadt, Germany. Fresh pork muscle (*M. longissimus dorsi*) (LD) was supplied by Ballyburden Meat Processors, Ballincollig, Co. Cork, Ireland. A spray-dried seaweed extract, containing laminarin (L) and fucoidan (F) (L/F extract) was manufactured by Bio Atlantis, Tralee, Co. Kerry, Ireland. The L/F extract was prepared from brown seaweed (*L. digitata*), using an acid extraction technique, the details of which are confidential. The composition of the L/F extract was 94.03% total solids, 68.6% ash, 0.64% protein, 9.3% laminarin, 7.8% fucoidan, and 8.3% mannitol. The ash component of the L/F extract was composed of 15 g/kg DM Ca, 10 g/kg DM Na, 10 g/kg DM K, 10 g/kg DM S, 250 mg/kg DM iodine, 250 mg/kg DM Fe, 20 mg/kg DM Cu and 50 mg/kg DM Zn. Tea catechins (TC) (81.43%) extracted from green tea was supplied by New Kinglong Natural Products Co. Ltd, Hunan, China.

2.2. Salt determination

The salt content of the L/F extract was measured using the mercuric nitrate titration method of Dubsky and Trtilek as described by Roberts (1936). The L/F extract (5 g) was dissolved in 40 ml of distilled water. Samples were filtered through Whatman No. 1 filter paper with glass wool into a 100 ml volumetric flask and made up to volume with distilled water. Filtrate (25 ml) plus 15 ml of 0.01 N HNO_3 and 1 ml of diphenylcarbazone indicator was titrated against 0.023 N $Hg(NO_3)_2$ standardised with 25 ml of 0.25% NaCl. A purple colour denoted the titration end-point. The salt percentage was calculated using the following equation:

$$\%\text{Salt} = \text{ml } Hg(NO_3)_2/5.$$

The salt content of the extract was 1% ± 0.1%. In the minced pork studies, a salt control (ST) was used to determine the effect of the salt contained in the L/F extract on the quality parameters examined. The addition of salt (50 mg/kg) to fresh minced pork (ST) was equivalent to the salt content of 0.5% L/F pork treatment.

2.3. Pork processing and packaging

M. longissimus dorsi (LD) muscles were trimmed of visible fat and connective tissue and minced twice through a plate with 4 mm holes (Model P114L, Talsa, Valencia, Spain). Following mincing, fresh pork was assigned to one of six treatments: untreated pork (Control); 0.005% salt (salt control) (ST); 0.1% tea catechin (positive lipid oxidation control) (TC); and pork plus increasing amounts of the L/F extract: 0.01% L/F, 0.1% L/F and 0.5% L/F. ST, TC and L/F were dissolved in water, immediately added to raw minced pork (5% v/w) and mixed vigorously.

Minced pork from each treatment was formed into patties (100 g portions) using a meat former (Ministeak burger maker, O.L. Smith Co. Ltd., Italy), placed in low oxygen permeable (<1 cm^3/m^2/24 h at STP) polystyrene/ethyl vinyl alcohol/polyethylene trays and using modified atmosphere packaging (MAP) technology, was flushed with 80% O_2:20% CO_2 using a vacuum-sealing unit (VS 100, Gustav Müller and Co. KG, Bad Homburg, Germany) equipped with a gas mixer (Witt-Gasetechnik GmbH and Co. KG, Witten, Germany). Trays were covered and heat-sealed using a low oxygen permeable (3 cm^3/m^2/24 h at STP) laminated barrier film with a polyolefin heat-sealable layer.

In the cooked pork study, minced pork patties (0.5% L/F) were placed on aluminium foil lined trays and cooked at 180 °C for 20 min in a fan-assisted convection oven (Zanussi Professional, Model 10 GN1/1, Conegliano, Italy) until an internal temperature of 72 °C was reached. Cooked patties were placed in MAP trays and flushed with 70% N_2:30% CO_2 and packaged as described previously. Fresh and cooked pork patties were stored for up to 14 days under fluorescent lighting conditions (approximately 660 lx) at 4 °C. The gas atmosphere (% O_2 and % CO_2) in the MAP was checked using a CheckMate 9900 (PBI-DanSensor, Denmark). The % N_2 was calculated by difference. Immediately after gas flushing, fresh pork MAP trays contained 77.15 ± 0.37% O_2 and 22.73 ± 0.33% CO_2 and the gas composition after 14 days was 67.75 ± 3.82% O_2 and 30.00 ± 3.87% CO_2. In cooked pork MAP trays, the gas composition was 67.46 ± 0.82% N_2 and 32.13 ± 0.49% CO_2 directly after gas flushing and 68.79 ± 0.14% N_2 and 31.10% ± 0.08% CO_2 after 14 days of storage.

2.4. Measurement of pH

Fresh minced pork samples (10 g) were homogenised for 1 min at 24,000 rpm in 90 ml distilled water using an Ultra Turrax T25 homogeniser (Janke and Kunkel, IKA-Labortechnik, GmbH and Co., Staufen, Germany). The pH of the pork homogenates was measured at 20 °C using a pH meter (Seven Easy portable, Mettler-Toledo GmbH, Schweizenbach, Switzerland). The pH of minced pork patties was recorded on days 1, 4, 7, 11 and 14 of storage.

2.5. Colour measurement

The surface colour was measured using a Konica Minolta CR-300 Chroma-Meter (Minolta Camera Co., Osaka, Japan). The Chroma-Meter consisted of a measuring head (CR-300), with an 8 mm diameter measuring area, a 2° standard observer, and a data processor (DP-301). The Chroma-Meter was calibrated on the CIE LAB colour space system using a white tile (D_c: $L = 97.79$, $a = -0.11$, $b = 2.69$). The 'L^*' value represents lightness and 'a^*' and 'b^*' values represent redness and yellowness, respectively. Colour measurements of fresh minced pork patties were recorded on days 1, 4, 7, 11 and 14 of storage.

2.6. Measurement of lipid oxidation

Lipid oxidation was measured using the 2-thiobarbituric acid assay of Siu and Draper (1978). The malondialdehyde content 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 pork. Lipid oxidation in fresh and cooked minced pork patties was measured on days 1, 4, 7, 11 and 14 of storage.

2.7. Microbiological analysis

Fresh minced pork (10 g) was transferred into stomacher bags, diluted with 90 ml of maximum recovery diluent and stomached for 3 min (Steward Stomacher 400 Lab Blender, London, UK) resulting in a 10^{-1} dilution used for analysis. Serial dilutions were prepared and 0.1 ml aliquots from each dilution were plated onto standard plate count agar (PCA) (Oxoid Ltd.). The plates were incubated at 30 °C for 48 h and at 4 °C for 10 days to determine mesophilic and psychrotrophic counts, respectively. Microbiological analysis of fresh minced pork patties from direct addition was carried out on days 1, 5, 8 and 12 of storage. Results were expressed as $\log_{10}$ cfu (colony forming units)/g pork.

2.8. Water holding capacity

Water holding capacity (WHC) was measured as described by Lianji and Chen (1989). Approximately 10 g of fresh minced pork was weighed into glass jars, covered with aluminium foil and heated in a water bath for 10 min at 90 °C. After heating, samples were carefully removed from each jar using forceps, wrapped in cheesecloth, and placed in 30 ml centrifuge tubes lined with cotton wool at the base of each tube. The samples were centrifuged (Beckman J2-21, Beckman Instruments Inc., CA, USA) at 13,440 ×g for 10 min at 4 °C. Following centrifugation, the cheesecloth was removed and samples were reweighed. Measurements of the moisture content (M) of pork samples were carried out on the Smart Trac5 rapid moisture/fat analyser (CEM Corporation). The percentage WHC was calculated using the following equation:

$$\%\text{WHC} = [1-[(\text{B}-\text{A})/(\text{B} \times \text{M})]] \times 100$$

where B denotes the weight of sample before heating; A, the weight of sample after heating and centrifuging; and M the % moisture of the sample (CEM). WHC was measured on days 2 and 7 of storage.

2.9. Texture profile analysis

Texture profile analysis (TPA) was carried out on cooked pork patties based on a method described by Bourne (1978). Cooked patties were cut into 20 mm pieces and axially compressed to 50% of their original height in a two-cycle compression test with an aluminium cylinder probe of 2 cm diameter using a texture analyser (TA.XT2i Texture Analyser, Stable Micro Systems, UK). Force time deformation curves were obtained using a 5 kN load cell applied at a cross speed of 50 mm/min. Attributes were calculated as follows: hardness (N), peak force required for first compression; springiness (mm), distance sample recovers after first compression; adhesiveness (N), the negative force area for the first bite representing the work necessary to pull the compressing plunger away from the sample; cohesiveness (dimensionless), ratio of positive force area during the second compression; gumminess (N), the product of hardness and cohesiveness; and chewiness (N × mm), the product of gumminess and springiness. TPA was carried out on days 2 and 7 comparable with sensory analysis measurement days.

2.10. Cook loss

The weight of minced pork patties was recorded before and after cooking. Patties were cooled for 1 h before re-weighing. Cook loss was measured on days 2 and 7 of storage and calculated using the following equation:

$$\%\text{cook loss} = [(\text{raw weight}-\text{cooked weight})/\text{raw weight}] \times 100.$$

2.11. Sensory evaluation

Sensory analysis ('visual' and 'eating quality') of fresh pork patties stored in 80% O_2:20% CO_2 was performed by 52 naïve assessors on days 2 and 7 of storage following the method of O'Sullivan, Byrne, and Martens (2003). On day 8, the bacterial count of the fresh pork patties (>6 $\log_{10}$ cfu/g) was considered too high for further sensory evaluation. 'Visual' sensory analysis descriptors were redness, brownness, drip, package quality, purchasing appeal, and overall acceptability. 'Eating quality' sensory analysis descriptors were appearance, tenderness, oxidation flavour, liking of flavour 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).

Pork patties were cooked for sensory analysis in a Zanussi oven at 180 °C for 20 min until an internal meat temperature of 72 °C was reached. Following cooking, patties were cooled and cut into 2 cm × 2 cm cubes and identified with random three-digit codes. Sample presentation order was randomised to prevent any flavour carryover effects (MacFie, Bratchell, Greenhoff, & Vallis, 1989). Prior to serving to panellists, pork samples were re-heated in a microwave for 10 s to release the meat odour and flavour. Sensory analysis was undertaken in the panel booths at the University sensory laboratory in accordance with the ISO (1988) international standard regulations. Assessors were also provided with water and crackers to cleanse their pallets between samples. Results for sensory analysis scores were measured in centimetres (cm) and scores were statistically analysed using ANOVA-Partial Least Squares Regression (APLSR). Results were presented as significance of regression coefficients, analysed by jack-knife testing.

2.12. Statistical analysis

All analyses were performed in duplicate and three independent experimental trials were carried out. A full repeated measures ANOVA was conducted to investigate the effects of L/F concentration, time and their interactions. L/F concentration represented the 'between-subjects' factor and the effect of time was measured using the 'within-subjects' factor. Tukey's test was used to adjust for multiple comparisons between treatment means. The analysis was carried out using SPSS 18.0 for Windows (SPSS, Chicago, IL, USA) software package.

'Visual' and 'eating quality' sensory data were analysed with ANOVA-Partial Least Squares Regression (APLSR) to process the mean data accumulated from the 52 test subjects. 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 APLSR models presented was determined to be 6 principal components. In these models assessor and session level effects were removed using level correction. The validated explained variance for the model constructed was −0.51% on day 2 and 6.27% on day 7 and the calibrated variance was 2.38% on day 2 and 8.97% on day 7. To derive significance indications for the relationships determined in the quantitative APLSR, regression coefficients were analysed by jack-knifing which is based on cross-validation and stability plots (Martens & Martens, 1999, 2001). All analyses were performed using the Unscrambler Software, version 9.8 (CAMO ASA, Trondheim, Norway).

3. Results and discussion

3.1. Colour stability of fresh minced pork

The '*L**' lightness values increased in fresh pork patties (0.01% L/F, 0.1% L/F, and 0.5% L/F) over the 14 day storage period and patties were unaffected by L/F extract concentration (Table 1). In a similar study, the addition of powdered *L. japonica* (1%, 3,% and 5%) decreased the lightness values of uncooked reduced fat pork patties due to the

brown and yellow pigments such as chlorophylls, phycophine, and xanthophylls present in the extract (Choi et al., 2012).

The '*a**' redness values of pork patties decreased as a function of storage time. The L/F extract (0.1% L/F, and 0.5% L/F) reduced the surface redness ('*a**' values) of pork patties on days 7, 11 and 14 of storage as a function of L/F concentration (Table 1). Similarly Cofrades et al. (2008) reported that the addition of dried *H. elongata*, *U. pinnatifida* and *P. umbilicalis* (2.5% and 5%) reduced the '*a**' redness values of a pork meat gel/emulsion in a concentration dependant manner.

Previous studies have linked oxymyoglobin oxidation and discoloration in meat to lipid oxidation, with an increase in one resulting in a similar increase in the other due to a number of proposed mechanisms (O'Grady, Monahan, & Brunton, 2001). At the highest level of L/F addition (0.5%), levels of lipid oxidation in pork patties were higher than the control on days 4, 7, 11 and 14 of storage (Table 2). Increased levels of lipid oxidation may subsequently promote oxymyoglobin oxidation which may explain the observed decreased surface redness values of pork patties in the present study.

In addition, decreased surface redness may be attributed to interactions between pork meat constituents and the added seaweed extract. Previous studies have indicated that polysaccharides can interact with proteins and it is generally accepted that electrostatic interactions occur between the anionic groups of a polysaccharide (seaweed polyanionic alginates) and the positively charged groups present in proteins (Imeson, Ledward, & Mitchell, 1977). Sulphated polysaccharides are capable of forming soluble complexes with globular proteins at pH values above the protein isoelectric point (Hill, Ledward, & Mitchell, 1998). Imeson et al. (1977) determined that the presence of the anionic polysaccharides altered the spectrum from that typical of metmyoglobin to one with a decreased absorption in the Soret (~400 nm) region; in the presence of pectate and alginate the Soret absorption also occurs at lower wavelengths. Therefore the dose-dependant decrease in '*a**' redness values, of pork patties may be partially attributed to interactions between polysaccharides (L/F) present in the extract and oxymyoglobin in pork meat.

In a previous study, a spray-dried L/F extract, identical to that used in the present study, did not affect the '*a**' redness values when incorporated into fresh pork via animal's diet (Moroney et al., 2012). Therefore the manner (dietary supplementation versus direct addition) by which seaweed extracts are added to meat appears to influence the mode of action of the bioactive components present.

The '*b**' yellowness values of fresh pork patties containing up to 0.1% L/F decreased over the 14 day storage period (Table 1). The addition of 0.5% L/F to fresh pork patties resulted in increased '*b**' yellowness values over time. Similarly, Cofrades et al. (2008) reported that dried *H. elongata* and *U. pinnatifida* (2.5% and 5%) increased '*b**' yellowness values in pork emulsion systems. In addition to chlorophyll, brown seaweeds contain phycophine, a brown pigment, and xanthophyll, a yellow pigment, which provides the seaweeds with a variety of shades in the yellow–dark chestnut range, including yellowish greens which can mask the green of chlorophyll (Cofrades et al., 2008). The change in the yellowness values of the pork patties in the present study may be attributed to the addition of natural colour pigments present in the L/F extract.

3.2. Lipid oxidation in fresh and cooked minced pork

In general, levels of lipid oxidation in fresh pork patties increased over time and followed the order: 0.5% L/F > ST > 0.1% L/F > Control > 0.01% L/F > TC (Table 2). No statistical difference was observed between L/F extract treatments (0.01% L/F and 0.1% L/F) and controls (control and TC) on any of the storage days. Levels of lipid oxidation were lowest in TC-containing pork patties. Previous studies have also demonstrated the potent antioxidant activity of TC in muscle foods (Tang, Kerry, Sheehan, Buckley, & Morrissey, 2001). In pork patties containing 0.5% L/F, the extract exerted the greatest lipid pro-oxidant activity on each measurement day. A pro-oxidant effect was also observed in pork patties containing salt (ST) at levels equivalent to 50 mg/kg, indicating that Na present in 0.5% L/F may be responsible for catalysis of lipid oxidation in fresh pork patties. Numerous studies have demonstrated the pro-oxidant properties of salt in muscle foods (Tang et al., 2001). In fresh meat, salt can promote the formation of hypervalent ferrylmyoglobin (or activated metmyoglobin), an initiator of lipid oxidation (Rhee & Ziprin, 2001).

The L/F extract also contained minerals such as iron (250 mg/kg DM) and copper (20 mg/kg DM) which are known to promote lipid oxidation in meat products (Bandy, Walter, Moon, & Davison, 2001; Decker & Xu, 1998; Rhee & Ziprin, 2001). Transition metals, in particular iron, initiate lipid oxidation either directly or indirectly by facilitating the generation of other initiating factors. Metals may also play a role in the propagation of lipid oxidation by catalysing the breakdown of lipid hydroperoxides and iron is considered a major catalyst of oxidative rancidity in meat (Ruiz, Perez-Vendrell, & Esteve-Garcia, 2000). Therefore, minerals present in the L/F extract may also be responsible for the catalysis of lipid oxidation in fresh pork patties.

In a previous study, the supplementation of porcine diets with a spray-dried L/F extract, identical to that used in the present study, decreased lipid oxidation in fresh pork LD muscle. It was concluded that antioxidant components of laminarin and fucoidan were deposited in LD muscle thereby enhancing lipid stability of pork

Table 1
Effect of L/F extract addition on surface lightness ('*L**' value), redness ('*a**' value) and yellowness ('*b**' value) values of fresh pork patties stored in modified atmosphere packs (80% O_2:20% CO_2) for up to 14 days at 4 °C.

Treatment	Parameter	Storage time at 4 °C, days				
		1	4	7	11	14
Control	Lightness	56.77 ± 2.25[a]	58.30 ± 3.12[a]	58.86 ± 1.58[a]	60.12 ± 1.49[a]	60.36 ± 2.19[a]
0.01% L/F	*L**	56.76 ± 2.19	58.20 ± 3.05	60.20 ± 0.99	61.10 ± 0.89	60.81 ± 2.17
0.1% L/F		56.66 ± 3.32	57.10 ± 3.83	59.15 ± 1.75	61.09 ± 1.11	60.88 ± 1.44
0.5% L/F		55.28 ± 2.31	56.24 ± 3.11	59.94 ± 1.28	62.19 ± 0.58	62.35 ± 1.45
ST*		56.19 ± 1.80	56.15 ± 1.51	57.88 ± 1.09	60.73 ± 2.50	61.94 ± 2.69
Control	Redness	8.78 ± 0.48[a]	6.87 ± 0.53[a]	4.89 ± 2.00[a]	3.29 ± 1.76[a]	2.40 ± 1.34[a]
0.01% L/F	*a**	8.68 ± 0.53	6.74 ± 0.51	4.72 ± 1.99	3.26 ± 1.86	2.14 ± 1.05
0.1% L/F		8.54 ± 0.54	6.86 ± 0.75	3.80 ± 1.92	1.56 ± 0.78	0.94 ± 0.67
0.5% L/F		7.79 ± 0.52	6.15 ± 0.77	2.47 ± 1.89	0.41 ± 0.72	−0.03 ± 0.72
ST		8.92 ± 0.31	7.33 ± 1.03	5.21 ± 2.65	1.85 ± 0.91	0.33 ± 0.68
Control	Yellowness	9.83 ± 0.34[a]	9.24 ± 0.20[a]	9.00 ± 0.16[a]	8.97 ± 0.48[a]	8.86 ± 1.02[a]
0.01% L/F	*b**	9.71 ± 0.33	9.24 ± 0.29	9.26 ± 0.34	9.22 ± 0.41	8.85 ± 0.66
0.1% L/F		9.82 ± 0.07	9.37 ± 0.29	9.08 ± 0.25	9.11 ± 0.67	9.27 ± 0.53
0.5% L/F		9.99 ± 0.27	9.81 ± 0.03	10.06 ± 0.51	10.21 ± 1.02	10.21 ± 0.40
ST		9.85 ± 0.29	9.36 ± 0.37	9.13 ± 0.67	9.76 ± 0.66	9.99 ± 0.39

[a] Within each parameter and storage day, mean values (±standard deviation) in the same column are not significantly different, $P > 0.05$.
* Salt control (ST), 0.005% salt.

Table 2
Effect of L/F extract addition on lipid oxidation (TBARS) of fresh and cooked pork patties stored in 80% O_2:20% CO_2, and 70% N_2:30% CO_2, respectively, for up to 14 days at 4 °C.

Treatment	Parameter	Storage time at 4 °C, days				
		1	4	7	11	14
Control	Fresh	0.062 ± 0.02[a]	0.134 ± 0.02[a]	0.119 ± 0.05[abc]	0.372 ± 0.23[abc]	0.374 ± 0.12[abc]
TC[†]		0.043 ± 0.02[a]	0.117 ± 0.04[a]	0.038 ± 0.02[b]	0.097 ± 0.06[b]	0.129 ± 0.05[b]
0.01% L/F		0.059 ± 0.01[a]	0.139 ± 0.01[a]	0.129 ± 0.07[abc]	0.341 ± 0.23[abc]	0.358 ± 0.13[abc]
0.1% L/F		0.062 ± 0.02[a]	0.155 ± 0.04[a]	0.207 ± 0.14[abc]	0.467 ± 0.30[abc]	0.582 ± 0.21[abc]
0.5% L/F		0.159 ± 0.04[b]	0.285 ± 0.08[b]	0.726 ± 0.47[c]	1.327 ± 0.72[c]	1.380 ± 0.77[abc]
ST[*]		0.090 ± 0.02[a]	0.116 ± 0.04[a]	0.282 ± 0.09[abc]	1.195 ± 0.64[c]	1.509 ± 0.93[c]
Control	Cooked	1.662 ± 0.07[a]	1.868 ± 0.29[a]	1.994 ± 0.36[a]	2.257 ± 0.06[a]	2.795 ± 0.01[a]
0.5% L/F		1.095 ± 0.30[b]	1.261 ± 0.16[b]	1.757 ± 0.55[a]	2.187 ± 0.48[a]	2.273 ± 0.13[b]

[abc] Within each parameter and storage day, mean values (±standard deviation) in the same column bearing different superscripts are significantly different, $P < 0.05$.
[†] Tea catechins (TC) (positive lipid oxidation control), 0.1%.
[*] Salt control (ST), 0.005% salt.

meat (Moroney et al., 2012). Dietary supplementation of L/F extract may serve to eliminate the effects of pro-oxidant components contained in the extract presumably due to porcine digestive and metabolic processes.

In contrast to the results reported in the present study, the addition of powdered *H. elongata*, *U. pinnatifida* and *P. umbilicalis* (2.5% and 5%) increased antioxidant activity in low-salt pork meat emulsion model systems. Antioxidant activity, determined using in vitro antioxidant assays (FRAP and TEAC), was attributed to polyphenolic compounds present in the seaweeds (López-López, Bastida, Ruiz-Capillas, Bravo, et al., 2009).

In cooked pork patties, lipid oxidation increased as a function of storage time. Pork patties containing L/F extract (0.5%) had significantly lower levels of lipid oxidation ($P < 0.05$) compared to the control on days 1, 4 and 14 of storage (Table 2). In a recent in vitro study, heat treatment (85 °C, 15 min) was reported to significantly improve the total phenolic, flavonoid, tannin and sugar contents in addition to the antioxidant activities (DPPH, metal ion chelating ability, H_2O_2 scavenging, and FRAP) of three edible Irish brown seaweeds (*Laminaria saccharina*, *L. digitata* and *H. elongata*) (Rajauria, Jaiswal, Abu-Ghannam, & Gupta, 2010). The spray-dried L/F extract examined in the present study is composed of polysaccharides which contain glucose. During cooking, reducing sugars such as glucose undergo non-enzymatic browning reactions such as the Maillard reaction (Manzocco, Calligaris, Mastrocola, Nicoli, & Lerici, 2000). The Maillard reaction has been associated with the formation of brown melanoidins (Maillard reaction products) with strong antioxidant capacity (Yilmaz & Toledo, 2005). The antioxidant activity of the L/F extract increased during cooking as demonstrated by the significant decrease in lipid oxidation of cooked pork patties, which may be attributed to Maillard reaction products formed during the cooking process which were not present in the fresh pork patties. Future studies are necessary to analyse the generation of Maillard reaction products during the cooking process.

3.3. Microbiology of fresh minced pork

In fresh pork patties, mesophilic and psychrotrophic total viable counts (TVC) increased during storage ranging from ~3.6 to 9.2 $\log_{10}$ cfu/g and ~5.0 to 9.8, respectively (Table 3). The TVC for 0.01% L/F, 0.1% L/F and 0.5% L/F were similar ($P > 0.05$) for all treatments, on each storage day, compared to controls indicating that L/F did not exert any antimicrobial activity in fresh pork patties. Similar findings were reported by Moroney et al. (2012) where supplementation of the L/F extract in porcine diets exhibited no effect on microbiological counts in fresh LD muscle.

Results from the present study are in contrast to previously reported studies where antimicrobial activity of *L. digitata* was assessed using in vitro test systems such as the growth inhibition assay and agar plate diffusion test (Dubber & Harder, 2008). In related studies, dried *U. pinnatifida* (3%) and dried *H. elongata* (5%) added to beef patties and pork frankfurters, respectively, initially resulted in increased levels of microbial growth compared to controls, presumably due to the product formulations rather than the seaweed (i.e. lower salt concentrations). However, trends were not statistically significant and no antimicrobial properties of the seaweed were detected over time (López-López, Cofrades, & Jiménez-Colmenero, 2009; López-López et al., 2010). While many in vitro studies on the bioactive compounds present in several seaweeds have demonstrated antimicrobial activity against a number of Gram positive and negative bacteria, no scientific literature exists demonstrating antibacterial activity of seaweed extracts in food products (Gupta & Abu-Ghannam, 2011).

3.4. pH, water holding capacity and cook loss of fresh minced pork

The pH of fresh pork patties decreased from ~5.7 to 5.6 over the 14 day storage period and was unaffected by the addition of the L/F extract. This pH range is comparable to values reported previously (5.8–5.4) for post-mortem muscle (Faustman & Cassens, 1990). Choi et al. (2012) reported a significant decrease ($P < 0.05$) in the pH of reduced-fat pork patties when powdered *L. japonica* (1%, 3,% and 5%) was added due to the presence of acid components such as alginic acid (pH: 2.3–2.8) in the seaweed extract.

The addition of L/F to fresh pork patties had no significant effect on the water holding capacity (WHC) values which ranged from 32.3 to 37.8% and 31.5–34.6% on days 2 and 7 of storage, respectively. Therefore, WHC decreased marginally as a function of storage time (Table 4). Fleury and Lahaye (1991) demonstrated that the physicochemical properties of seaweeds are determined by the chemical structure of the constituent polysaccharides present in the algae. Previous studies on physicochemical properties of seaweeds report a positive correlation between water retention and swelling capacity (Rupérez & Saura-Calixto, 2001). Therefore, meat products with added seaweed or seaweed extracts, may have improved water and

Table 3
Effect of L/F extract addition on microbial status (mesophilic and psychrotrophic) of fresh pork patties stored in modified atmosphere packs (80% O_2:20% CO_2) for up to 12 days at 4 °C.

Treatment	Temp	Storage time at 4 °C, days			
		1	5	8	12
Control	30 °C	5.19 ± 0.08[a]	6.79 ± 0.37[a]	8.79 ± 0.59[a]	9.01 ± 0.46[a]
0.01% L/F		5.02 ± 0.28	6.84 ± 0.45	8.90 ± 0.61	9.01 ± 0.62
0.1% L/F		5.01 ± 0.24	6.84 ± 0.23	8.82 ± 0.62	8.89 ± 0.74
0.5% L/F		5.16 ± 0.06	6.85 ± 0.22	8.86 ± 0.69	9.22 ± 0.58
Control	4 °C	6.30 ± 0.34[a]	8.17 ± 0.44[a]	9.32 ± 0.12[a]	9.46 ± 0.26[a]
0.01% L/F		6.26 ± 0.20	8.16 ± 0.55	9.26 ± 0.32	9.31 ± 0.27
0.1% L/F		6.37 ± 0.27	8.19 ± 0.64	9.48 ± 0.18	9.23 ± 0.39
0.5% L/F		6.37 ± 0.26	8.33 ± 0.57	9.63 ± 0.19	9.75 ± 0.09

[a] Within each parameter and storage day, mean values (±standard deviation) in the same column are not significantly different, $P > 0.05$.

Table 4
Effect of L/F extract addition on cook loss and water holding capacity (WHC) of fresh pork patties stored in modified atmosphere packs (80% O_2:20% CO_2) at 4 °C.

Treatment	Storage time at 4 °C (d)			
	Cook loss		WHC	
	2	7	2	7
Control	31.99 ± 0.93[a]	32.20 ± 0.94[a]	35.09 ± 1.72[a]	33.50 ± 3.37[a]
0.01% L/F	32.29 ± 1.23	32.57 ± 0.58	37.79 ± 1.62	33.87 ± 2.83
0.1% L/F	32.05 ± 0.31	31.95 ± 1.53	36.55 ± 1.82	32.08 ± 1.67
0.5% L/F	31.36 ± 1.53	29.26 ± 1.31	37.26 ± 1.40	34.63 ± 3.60
ST*	30.61 ± 0.77	29.01 ± 4.00	32.27 ± 4.10	31.53 ± 4.72

[a] Within each parameter and storage day, mean values (±standard deviation) in the same column are not significantly different, $P > 0.05$.
* Salt control (ST), 0.005% salt.

fat binding properties (López-López, Cofrades, & Jiménez-Colmenero, 2009).

Cook loss was unaffected by the addition of the L/F extract and ranged from 30.6 to 32.3% and 29.0–32.6% on days 2 and 7 of storage, respectively (Table 4). In a previous study, the cook loss of reduced-fat pork patties containing powdered *L. japonica* (1%, 3,% and 5%) was significantly lower than that of the control. Reduced cook loss was attributed to dietary fibres such as alginate and laminarin, which have high water holding and binding capacities (Choi et al., 2012). The functional properties of agar, alginates and carrageenans in food products have been well documented in the scientific literature. Scientific reports on laminarin and fucoidan focus primarily on biological activity (Elleuch et al., 2011; Thebaudin, Lefebvre, Harrington, & Bourgeois, 1997; Venugopal, 2008). The lack of effect of the L/F extract in the present study may be due to the low concentration at which the extract was added to the fresh pork patties.

3.5. Texture profile analysis of fresh minced pork

Hardness levels decreased between storage days 2 and 7 for all treatments. General trends indicated that hardness increased with the addition of L/F in a dose-dependent manner on day 2 of storage compared to the controls (Table 5). In general, the addition of various types of fibres (soy, wheat, cereal or fruit) increased hardness of cooked meat emulsions (Fernández-Ginés, Fernández-Lopez, Sayas-Barberá, & Pérez-Alvarez, 2005). The hardness of gel/emulsion pork meat systems and frankfurters increased due to the addition of dried *H. elongata*, *U. pinnatifida*, and *P. umbilicalis* (2.5 and 5%), and *H. elongata* (5.6%), respectively (Cofrades et al., 2008; López-López, Bastida, Ruiz-Capillas, Bravo, et al., 2009). The L/F extract did not have a significant effect on springiness or cohesiveness, on either assessment day, relative to the controls. General trends on days 2 and 7 of storage, while not statistically significant, indicated that gumminess and chewiness parameters in cooked pork patties increased at L/F levels up to 0.1% (Table 5).

The proportion, composition (soluble and insoluble fractions) and characteristics (including particle size) of dietary fibre greatly influence physicochemical properties exerted in meat products (Gómez-Ordóñez et al., 2010). The texture of pork products may be enhanced by seaweed extracts depending on the amount and type of dietary fibre present in the extract. Conflicting studies demonstrated no beneficial effects of dietary fibre on pork texture (Cofrades et al., 2008; López-López et al., 2010). The thickening and gelling properties and the water-retention ability of soluble fibres contribute to the stabilisation of the structure of foods (dispersions, emulsions and foams) by modifying rheological properties of the continuous phase. Insoluble fibres can also influence food texture due to their water-retention ability and swelling properties (Thebaudin et al., 1997). The direct addition of the L/F extract (composed of 17.1% soluble fibres) at low levels did not significantly affect the textural parameters of the fresh pork patties in the present study which may be due to the very low level and type of soluble fibres added to the fresh pork patties.

3.6. Sensory evaluation of fresh and cooked minced pork

In 'visual' sensory analysis of minced pork patties (Table 6), the control and 0.01% L/F were significantly ($P < 0.01$) positively correlated to redness while 0.5% L/F was significantly ($P < 0.001$) positively correlated to brownness on day 7 of storage. This is in agreement with instrumental '*a**' redness values, where a negative effect was exerted by L/F at the higher concentrations. Similarly, powdered *L. japonica* (1%, 3,% and 5%) was found to significantly ($P < 0.05$) decrease the colour score of reduced-fat pork patties due to the dark brown colour of the seaweed powder (Choi et al., 2012). On day 7, 0.01% L/F and the control were significantly ($P < 0.01$) positively correlated to purchasing appeal and overall acceptability while 0.5% L/F was significantly ($P < 0.001$) negatively correlated to purchasing appeal and overall acceptability. Therefore, the 'visual' sensory properties of pork patties were negatively affected by the addition of 0.5% L/F extract, but at the lower level (0.01% L/F), were still acceptable to sensory panellists.

No significant trends were observed for 'eating quality' sensory analysis with the exception of tenderness, which was significantly ($P < 0.05$) negatively correlated with 0.5% L/F on day 7 of storage (Table 6). However, no significant effect on the TPA from the L/F extract was observed. This indicated that the L/F extract can be incorporated at low levels (0.01%–0.1%) without detrimentally affecting the texture. No significant correlations were observed for overall acceptability and trends indicated positive directional correlations for control, 0.01% L/F and 0.1% L/F. In a previously reported study, the addition of dried *U. pinnatifida* (3.3%) did not negatively affect the sensory properties of low-salt and low-fat beef patties in comparison with the control (López-López et al., 2010). Choi et al. (2012) reported the addition of powdered *L. japonica* (1%, 3,% and 5%) to reduced-fat pork patties demonstrated significantly ($P < 0.05$) higher overall acceptability scores indicating foods containing seaweed had better sensory scores when compared to the controls. In the present study, sensory panellists were unable to distinguish between the control and pork patties containing low levels of the L/F extract.

Table 5
Effect of L/F extract addition on texture profile analysis (TPA) of fresh pork patties stored in modified atmosphere packs (80% O_2:20% CO_2) at 4 °C.

Treatment	Day	Hardness	Springiness	Cohesiveness	Gumminess	Chewiness
Control	2	36.56 ± 17.30[a]	0.86 ± 0.02[a]	0.55 ± 0.02[a]	20.12 ± 9.30[a]	17.12 ± 7.54[a]
0.01% L/F		38.68 ± 08.80	0.85 ± 0.02	0.55 ± 0.01	21.37 ± 4.81	18.14 ± 3.72
0.1% L/F		52.34 ± 16.00	0.86 ± 0.02	0.55 ± 0.01	28.55 ± 8.31	24.54 ± 6.61
0.5% L/F		44.20 ± 17.03	0.84 ± 0.04	0.56 ± 0.02	24.33 ± 8.90	20.26 ± 7.12
Control	7	27.04 ± 06.62[a]	0.87 ± 0.02[a]	0.56 ± 0.02[a]	15.02 ± 4.03[a]	13.12 ± 3.84[a]
0.01% L/F		24.21 ± 06.67	0.87 ± 0.04	0.56 ± 0.01	13.51 ± 3.48	11.62 ± 2.66
0.1% L/F		31.76 ± 10.50	0.85 ± 0.04	0.55 ± 0.02	17.65 ± 5.94	15.15 ± 5.32
0.5% L/F		25.33 ± 02.03	0.87 ± 0.03	0.55 ± 0.02	14.06 ± 1.21	12.20 ± 1.16

[a] Within each parameter and storage day, mean values (± standard deviation) in the same column are not significantly different, $P > 0.05$.

Table 6
Significance of regression coefficients (ANOVA values) for minced pork patties as derived by jack-knife uncertainty testing.

Parameter	Storage time, days/treatment							
	Control		0.01% L/F		0.1% L/F		0.5% L/F	
	2	7	2	7	2	7	2	7
Visual sensory analysis								
Redness	−0.47	0.000***	0.28	0.003**	0.42	−0.06	−0.004**	−0.000***
Brownness	−0.51	−0.000***	−0.34	−0.002**	−0.45	0.06	0.08	0.000***
Drip	0.96	−0.24	0.97	−0.24	0.96	0.35	−0.96	0.21
Packaging quality	−0.76	0.05*	−0.78	0.05*	−0.77	−0.18	0.76	−0.03*
Purchasing appeal	0.57	0.000***	0.22	0.003**	0.28	−0.09	−0.02*	−0.000***
Overall acceptability	0.57	0.000***	0.25	0.002**	0.27	−0.08	−0.03*	−0.000***
Eating quality sensory analysis								
Appearance	−0.82	0.49	−0.81	0.50	−0.81	−0.55	0.81	−0.47
Tenderness	−0.46	0.10	−0.72	0.09	−0.77	−0.30	0.68	−0.04*
Oxidation flavour	−0.55	0.15	−0.34	0.14	−0.39	−0.25	0.13	−0.14
Liking of flavour	0.80	−0.97	0.71	−0.97	0.60	0.97	−0.71	0.97
Overall acceptability	0.75	0.57	0.60	0.58	0.36	−0.63	−0.59	−0.56

* 95% significance, $P < 0.05$.
** 99% significance, $P < 0.01$.
*** 99.9% significance, $P < 0.001$.

4. Conclusions

Addition of a seaweed extract containing polysaccharides (soluble dietary fibres) did not enhance quality parameters of the fresh minced pork patties. The L/F extract exerted a pro-oxidant effect on lipid oxidation over time attributed to the pro-oxidant components (sodium, copper and iron) present in the extract. Decreased lipid oxidation observed in cooked pork patties containing the L/F extract (0.5%) provided evidence that heating can enhance the antioxidant capacity of seaweed extracts in muscle foods and improve quality parameters possibly do to the formation of brown melanoidins (Maillard reaction products) with antioxidant functionality. The L/F extract at a level of 0.01% can be incorporated without adversely affecting the colour, lipid oxidation, texture or sensorial acceptance of pork patties. Further research is necessary to examine the effects of more refined or purified laminarin and fucoidan extracts in meat products.

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Article

Seaweed Polysaccharides (Laminarin and Fucoidan) as Functional Ingredients in Pork Meat: An Evaluation of Anti-Oxidative Potential, Thermal Stability and Bioaccessibility

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Abstract: The anti-oxidative potential of laminarin (L), fucoidan (F) and an L/F seaweed extract was measured using the DPPH free radical scavenging assay, in 25% pork (*longissimus thoracis et lumborum* (LTL)) homogenates (TBARS) (3 and 6 mg/mL) and in horse heart oxymyoglobin (OxyMb) (0.1 and 1 mg/mL). The DPPH activity of fresh and cooked minced LTL containing L (100 mg/g; L_{100}), F_{100} and $L/F_{100,300}$, and bioaccessibility post *in vitro* digestion (L/F_{300}), was assessed. Theoretical cellular uptake of antioxidant compounds was measured in a transwell Caco-2 cell model. Laminarin displayed no activity and fucoidan reduced lipid oxidation but catalysed OxyMb oxidation. Fucoidan activity was lowered by cooking while the L/F extract displayed moderate thermal stability. A decrease in DPPH antioxidant activity of 44.15% and 36.63%, after 4 and 20 h respectively, indicated theoretical uptake of L/F antioxidant compounds. Results highlight the potential use of seaweed extracts as functional ingredients in pork.

Keywords: laminarin; fucoidan; seaweed extract; *in vitro* digestion; bioaccessibility; pork

1. Introduction

Seaweed polysaccharides (laminarin and fucoidan) isolated from the cell walls of brown seaweed (*Laminaria digitata*) possess immunomodulatory, anti-inflammatory, antiviral, antitumor, antithrombotic anticoagulant and antioxidant bioactivities [1,2]. Structurally, laminarin is composed of β-(1,3)-linked glucose containing large amounts of sugars and a low fraction of uronic acids. Two types of polymeric chains are present in laminarin, G-chains with glucose at the end and M-chains with mannitol as the terminal reducing end [3]. The antioxidant activity of laminarin has been linked to molecular structure, degree and length of branching and the monosaccharide constituents [4]. The structure of fucoidan consists mainly of α(1,3)-linked L-fucopyranose residues with sulphates at the C-2 position [5]. Distinct conclusions regarding chemical structures of fucoidans are often difficult to formulate due to structural heterogeneity and lack of regularity in fucoidan molecules [6]. Sulphate content, degree of sulphation and molecular weight are often attributed as factors influencing the antioxidant activity of fucoidan [7].

A wide range of analytical techniques (e.g., HPLC, ATR-FTIR and NMR spectroscopy) may be used to characterise and quantify structurally complex polysaccharides, such as laminarin and fucoidan, present in seaweeds [8]. Such techniques can involve detailed and time consuming extraction, preparation and sample clean-up procedures, depending on the parent seaweed material or the matrix in which the compounds of interest (polysaccharides) are contained [9]. *In vitro* antioxidant assays (e.g., FRAP, ABTS, ORAC and DPPH free radical scavenging activities) are frequently used to assess the antioxidant activity and potency of plant extracts [10]. The DPPH assay (based on a quick electron transfer reaction, followed by a slower hydrogen transfer reaction) is a simple, rapid, sensitive and reproducible index of antioxidant activity [11]. DPPH free radical scavenging activity of seaweed extracts, including laminarin and fucoidan, has been reported for a number of seaweed species [11,12].

The addition of antioxidant compounds to muscle foods (via the animals' diet or direct addition) in order to enhance meat quality and shelf-life has attracted much research attention in recent years. Previous research indicated that functional ingredients, such as laminarin and fucoidan, have beneficial effects pre-(animal health) [13] and post-slaughter (meat quality) [14]. Moroney *et al.* [15] reported that the addition of seaweed extracts, containing laminarin and fucoidan, to pig diets, resulted in lower levels of lipid oxidation in fresh pork steaks. However, direct addition of the same seaweed extract, promoted lipid oxidation and decreased the surface redness of fresh pork patties [16]. Catalysis of lipid oxidation was linked to the presence of salt and minerals in the seaweed extract. Increased discolouration (oxymyoglobin oxidation) was attributed to the effect of oxidising lipids and potential interactions between seaweed polysaccharides and oxymyoglobin. The anti- and pro-oxidative activity of laminarin and fucoidan on lipid and oxymyoglobin oxidation processes will be further examined in the present study.

The chemical structure of plant cell wall polysaccharides (e.g., cellulose, pectin substances, inulin and gums) and other associated non-carbohydrate components (*i.e.*, resistant protein) can be sensitive to chemical, mechanical, thermal and enzymatic processing [17]. Therefore the consequence of cooking on the potential bioactivity of laminarin and fucoidan in a meat matrix should be considered when formulating a functional meat product [18]. Cooking may sometimes improve the antioxidant activity of plant based materials due to the formation of other antioxidant components such as Maillard reaction products (MRPs) [19]. MRPs have been reported to possess antiradical activity including inhibition of the DPPH, oxygen peroxyl and hydroxyl radicals as well as copper and Fe^{2+} chelators [20]. In a

previous study, Moroney *et al.* [16] reported a reduction in lipid oxidation of cooked minced pork patties containing laminarin and fucoidan which was attributed partially to the cooking process and the formation of MRPs which were not present in the fresh pork patties.

The digestion process may influence the bioactivity and bioaccessibility of laminarin and fucoidan. Bioaccessibility is defined as the fraction of a compound transferred from the food matrix during digestion, and thus made accessible for intestinal absorption and cellular uptake [21]. *In vitro* digestion models provide a useful alternative to animal and human models and simulate the digestion process of the human gastrointestinal tract (GIT). Cell culture models, in particular the Caco-2 cell culture model, have been widely utilised as part of *in vitro* digestion models as a predictive tool for the absorption of bioactive compounds from foods [22].

Studies on the anti-oxidative potential of seaweed polysaccharides in meat products are limited and merit investigation. Furthermore, the literature lacks information regarding the bioaccessibility of seaweed polysaccharides in meat products after cooking and post digestion. The initial objective of this study was to profile the antioxidant activity of laminarin (L), fucoidan (F) and a seaweed extract containing L and F, using the DPPH free radical scavenging assay. The antioxidative potential of L, F and L/F was further examined in fresh pork *longissimus thoracis et lumborum* (LTL) homogenates and in commercial horse heart oxymyoglobin. The DPPH radical scavenging and thermal stability of L, F and L/F in cooked pork patties was assessed. Finally cooked pork patties were subjected to an *in vitro* digestion procedure to determine the effects of digestion on the antioxidant potential of L, F and L/F and L/F digestates were examined in a transwell Caco-2 cell model to assess theoretical cellular uptake of antioxidant components of L/F.

2. Results and Discussion

2.1. Free Radical Scavenging Activity of Seaweed Polysaccharides (L, F and L/F)

In general, the DPPH free radical scavenging activity of seaweed polysaccharides increased over 20 h and followed the order: Trolox > F_1 > L/F_3 > L/F_1 > L_{10} ≈ L_1 (Table 1). DPPH free radical scavenging activity of L/F increased as a function of concentration. The DPPH free radical scavenging activities reported for L_1 and L_{10} were comparable to previously reported values (1.4%–5.3%) for laminarin extracted from *Laminaria digitata* at concentrations ranging from 0.125 to 1.0 mg/mL [12]. The DPPH free radical scavenging activity of F_1 (66.13%) after 1 h in the present study was similar to the inhibition of the DPPH radical (55.22%) after 30 min by fucoidan (1 mg/mL) from Sigma reported by Mak *et al.* [7].

Limited research suggests that carbohydrate polymers such as β-glucans (laminarin) possess free radical scavenging activity, however the addition of high levels of β-glucans is often necessary before radical scavenging activity is observed [23,24]. At concentrations of 20–200 mg/mL (higher than those used in the present study) a 1,3 β-D-glucan enriched extract from cereal grains demonstrated 25%–80% inhibition of the DPPH radical [23]. The mechanism of antioxidant action of β-D-glucans against free radicals is still not well understood, but a number of theories exist [25]. Tsiapali *et al.* [24] reported enhanced antioxidant activity of laminarin polymers over monomeric units due to greater ease of abstraction of anomeric hydrogen from one of the internal monosaccharide units rather than from the

reducing end. In the present study, laminarin exhibited weak radical scavenging activity which may be due to the level examined.

Table 1. Free radical scavenging activity (DPPH) of L, F and L/F for up to 20 h at ~20 °C.

Incubate	Time, h		
	1	4	20
L_1 *	1.09 ± 0.92 ^{ab}	1.39 ± 0.96 ^{ab}	1.64 ± 1.30 ^{ab}
L_{10}	1.55 ± 1.21 ^{b}	2.72 ± 1.77 ^{b}	3.16 ± 2.72 ^{b}
F_1	66.13 ± 0.32 ^{c}	76.48 ± 0.30 ^{c}	90.68 ± 0.55 ^{c}
L/F_1	35.43 ± 2.04 ^{d}	47.35 ± 1.79 ^{d}	69.51 ± 1.37 ^{d}
L/F_3	56.18 ± 1.01 ^{e}	68.40 ± 0.89 ^{e}	78.41 ± 0.21 ^{e}
Trolox	95.89 ± 0.08 ^{f}	95.92 ± 0.14 ^{f}	95.76 ± 0.48 ^{f}

* Subscripts 1, 3 and 10 denote concentrations in mg/mL; $^{a–f}$ Within each storage time, mean values (± standard deviation) in the same column bearing different superscripts are significantly different, $p < 0.05$.

For some antioxidants, such as Trolox, the reaction with DPPH is rapid while other compounds may react more slowly [26]. The ability of seaweed extracts to quench free radicals is known to take place over longer periods of time compared to rapid acting synthetic antioxidants such as butylated hydroxyanisole (BHA) [27,28]. Slower reacting compounds are hypothesised to have a more complex reaction mechanism involving one or more secondary reactions in quenching the DPPH radical [10]. In the present study, after 20 h, the DPPH free radical scavenging activity of F_1 was equivalent (although statistically lower) to the positive control (Trolox), and significantly ($p < 0.05$) higher than both L_1 and L_{10}. Therefore the ability of an antioxidant to reduce and quench free radicals over a longer period of time may have benefits for extending the shelf-life of processed foods [28].

2.2. Effect of Seaweed Polysaccharides on Lipid Oxidation in Pork Muscle Model Systems

In vitro antioxidant assays (e.g., the DPPH assay) highlight the potential antioxidant activities of compounds but may not accurately predict activity in complex test systems such as muscle foods. To further investigate antioxidant activities of L, F and L/F, pork meat homogenates were subjected to iron/ascorbate ($FeCl_3$/sodium ascorbate)-induced lipid oxidation. Compared to the control, after 4 h at 4 °C, lipid oxidation significantly increased ($p < 0.05$) in the pork meat homogenates with the addition of pro-oxidants (Figure 1). No difference was observed for L_3 and L_6 compared to the control. Similarly no inhibition of lipid oxidation by laminarin, at levels comparable to those in the present study (3 mg/mL), was observed in a linoleic acid emulsion system [25]. F_3 and F_6 significantly decreased ($p < 0.05$) levels of lipid oxidation in pork meat homogenates. Trends indicated that levels of lipid oxidation in L/F_3 and L/F_6 were lower than the control (with pro-oxidants) although results were not statistically significant. In a previous study, Moroney *et al.* [16] reported that salt and minerals, present in the L/F extract, may have promoted lipid oxidation in fresh pork patties. Minerals and salt present in L/F_3 and L/F_6 may have counteracted the antioxidant activity of other constituents in the extract, thus impeding ability to significantly enhance lipid stability in the pork meat homogentates (Figure 1).

Structurally laminarin does not contain sulphate groups, which reportedly increases the antioxidant activity of fucoidan [29]. Sulphate groups can enhance the steric hindrance between polymer chains in polysaccharides leading to a more ordered and expanded conformation thus improving homogeneity in aqueous solution [30]. Lower molecular weight polysaccharides are often linked to increased free radical scavenging ability, presumably due to a non-compact structure which may allow more available sulphate and hydroxyl groups react with free radicals [9]. However, this was not observed for L in the present study indicating that even at low molecular weight, the structure in the presence of pork meat was unable to inhibit lipid oxidation, similar to the lack of DPPH free radical scavenging activity observed in Section 2.1 (Table 1).

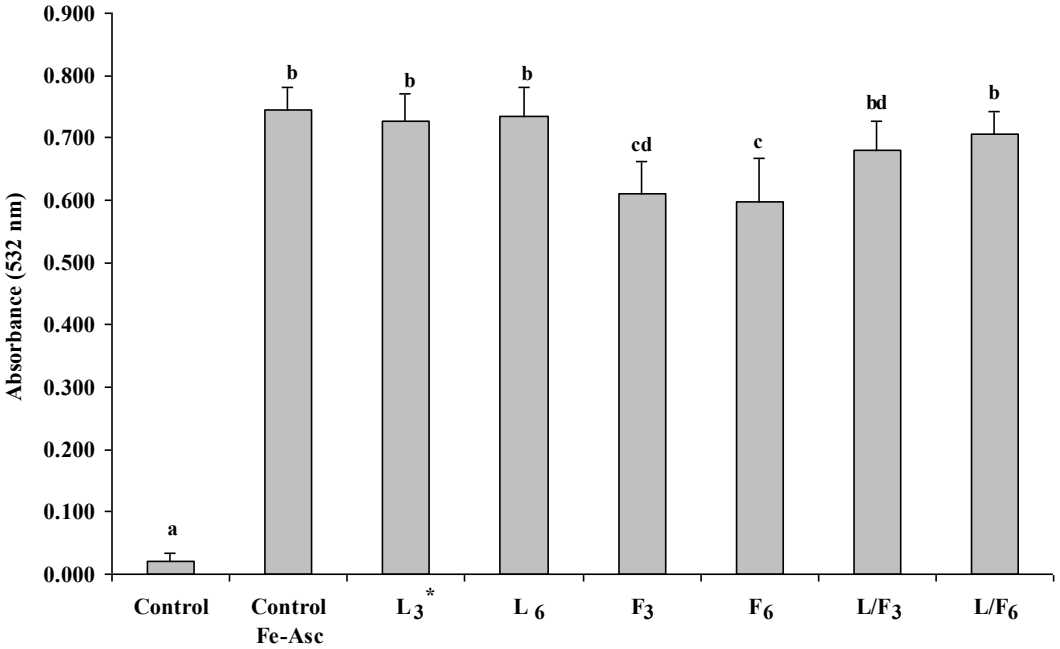

Figure 1. Lipid oxidation in 25% *longissimus thoracis et lumborum* (LTL) pork muscle homogenates following the addition of L, F or L/F and storage for up to 4 h at 4 °C. * Subscripts 3 and 6 denote concentrations in mg/mL. ^{abcd} Mean values (± standard deviation error bars) bearing different superscripts are significantly different, $p < 0.05$.

In general, it is accepted that natural antioxidants scavenge free oxygen-centered radicals via two major mechanisms, hydrogen atom transfer (HAT) reactions and electron transfer (ET) reactions. Yan *et al.* [30] suggested the HAT reaction is more likely to occur in neutral polysaccharides, such as laminarin, while the ET is the probable mechanism in acidic polysaccharides, like fucoidan where the negative charge of the sulphate groups plays a large part in the radical scavenging activity. In the present study, fucoidan is most likely responsible for the antioxidant activity observed by the L/F extract in the pork meat homogenates presumably due to ET reactions between the sulphate groups and the free radicals in the pork meat homogenates.

2.3. Effect of Seaweed Polysaccharides on Oxymyoglobin Oxidation

Oxymyoglobin oxidation (represented by a reduction in OxyMb, %) increased during storage for up to 8 days at 4 °C (Table 2). $L_{0.1}$ and L_1 had no influence on OxyMb oxidation, however $F_{0.1}$ and F_1

significantly ($p < 0.05$) enhanced OxyMb oxidation compared to the control in a dose dependant manner on days 4 and 8 of storage. Similarly, a significant increase ($p < 0.05$) in OxyMb oxidation was observed for $L/F_{0.1}$ and L/F_1. The presence of metmyoglobin is characterised by an increased absorption at ~628 nm [31] which is evident in the spectral scan for OxyMb alone and OxyMb + F_1 (Figure 2). At the wavelengths examined, no spectral shift in the presence of F_1 was observed.

Table 2. Oxymyoglobin (OxyMb) oxidation (represented by a reduction in OxyMb) following the addition of L, F or L/F and storage for up to 8 d at 4 °C.

Incubate	time, d		
	0	4	8
Control	76.53 ± 2.28 ^{a}	59.92 ± 2.30 ^{a}	54.46 ± 2.02 ^{a}
$L_{0.1}$ *	76.57 ± 2.31 ^{a}	59.68 ± 2.14 ^{ab}	54.00 ± 2.50 ^{a}
L_1	76.59 ± 2.73 ^{a}	58.11 ± 3.12 ^{abc}	52.51 ± 2.75 ^{a}
$F_{0.1}$	74.73 ± 2.54 ^{ac}	53.44 ± 2.44 ^{bd}	45.42 ± 2.56 ^{b}
F_1	67.55 ± 2.50 ^{b}	32.95 ± 2.00 ^{e}	21.71 ± 1.34 ^{c}
$L/F_{0.1}$	74.93 ± 2.06 ^{ad}	52.91 ± 2.44 ^{cd}	44.95 ± 2.82 ^{b}
L/F_1	69.03 ± 2.78 ^{bcd}	39.01 ± 1.90 ^{e}	28.78 ± 2.25 ^{d}

* Subscripts 0.1 and 1 denote concentrations in mg/mL; ^{abcde} Within each storage time, mean values (± standard deviation) in the same column bearing different superscripts are significantly different $p < 0.05$.

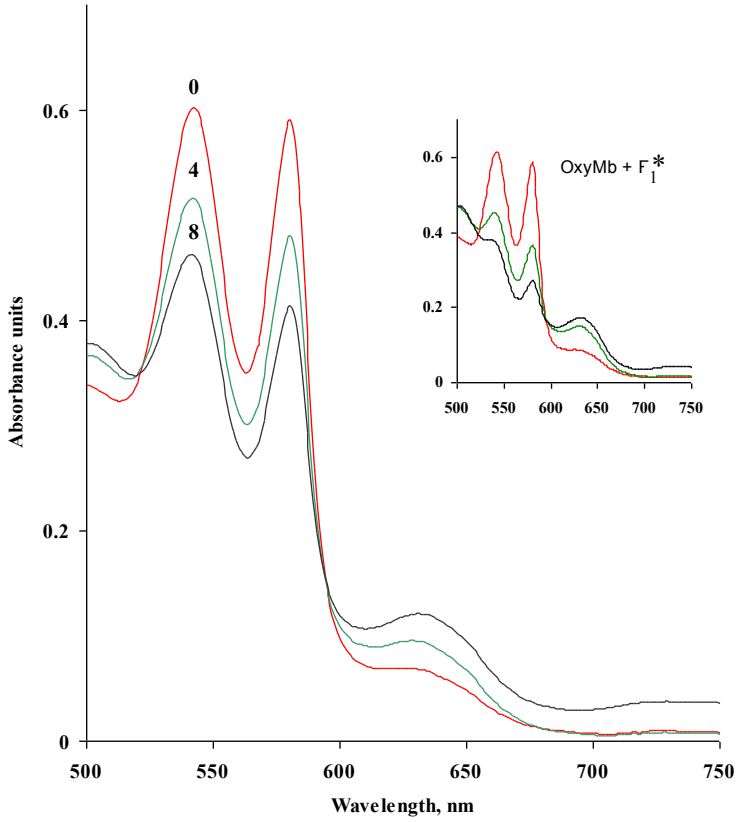

Figure 2. Absorbance spectra of oxymyoglobin (OxyMb) alone and following the addition of F_1 (* Subscript 1 denotes concentration in mg/mL) and storage for up to 8 days at 4 °C.

The exact mechanism by which fucoidan promotes OxyMb oxidation is unclear. The ability of fucoidan to bind to proteins such as antithrombin (a glycoprotein) and bovine serum albumin (a

globular protein) has previously been linked to molecular weight as well as the sulphation patterns of the polysaccharide [32–34]. Generally, interactions between anionic polysaccharides and positively charged OxyMb have been reported to be electrostatic in nature due to opposing charges [35].

Similarly, Satoh *et al.* [36] demonstrated that oxidation of OxyMb was initiated via nucleophilic attack at the iron (II) centre of OxyMb by a water molecule with strong proton assistance from the distal histidine, or a hydroxide anion (OH^-). These reactions can cause irreversible displacement of bound dioxygen from OxyMb resulting in the formation of ferric metmyoglobin and generation of the superoxide anion. In the present study, the anionic sulphate groups of fucoidan potentially enhanced the oxidation of OxyMb through the nucleophilic displacement mechanism described above.

2.4. Effect of Cooking on the DPPH Free Radical Scavenging Activity of Seaweed Polysaccharides in Pork Meat

Statistical analysis indicated that the DPPH free radical scavenging of L, F and L/F in the presence of fresh minced LTL ($F_{100} > L/F_{300} \approx L/F_{100} \approx L_{100}$) followed a similar pattern to the DPPH free radical scavenging activities of seaweed polysaccharides reported in Section 2.1. L_{100} DPPH free radical scavenging was similar to the control before and after cooking (Figure 3). The DPPH free radical scavenging activity of F_{100} significantly ($p < 0.05$) decreased after cooking. Thermal processing is known to modify the physicochemical properties of plant cell wall polysaccharides [17]. The DPPH free radical scavenging activities of fresh and cooked L/F_{100} and L/F_{300} were similar indicating moderate thermal stability of the L/F extract. Similarly, Moroney *et al.* [14] reported low to moderate thermal stability of L/F in cooked minced pork patties from pigs fed the L/F extract for 3 weeks pre-slaughter.

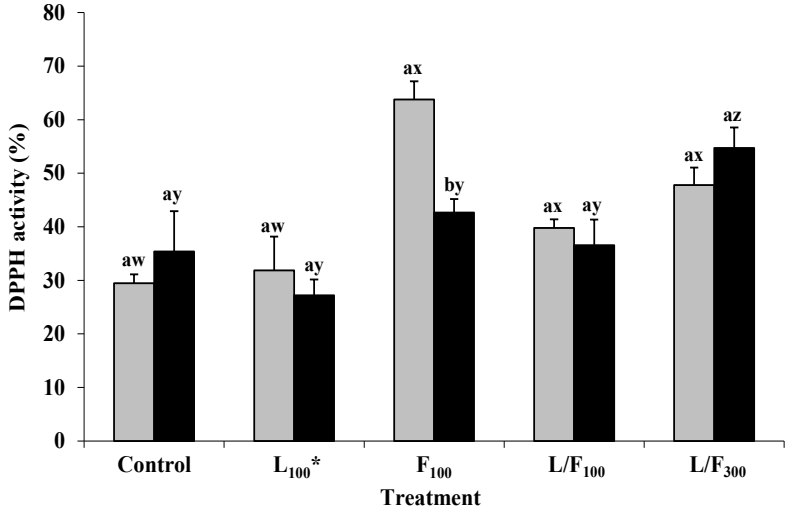

Figure 3. Free radical scavenging activity (DPPH) of L, F or L/F in fresh and cooked minced *longissimus thoracis et lumborum* (LTL) pork muscle stored for 20 h at ~20 °C. ***** Subscripts 100 and 300 denote concentrations in mg/g. [ab] Within each treatment, mean values (± standard deviation error bars) bearing different superscripts are significantly different, $p < 0.05$. Comparing [wx] fresh and [yz] cooked LTL pork muscle treatments to their respective controls, mean values bearing different superscripts are significantly different, $p < 0.05$. (□), fresh; (■), cooked.

L/F_{300} significantly ($p < 0.05$) enhanced the DPPH free radical scavenging activity of cooked minced LTL compared to the control (Figure 3). Similarly, Prabhasankar *et al.* [37] reported an increase in DPPH free radical scavenging activity of cooked pasta with the addition of a brown seaweed (*Undaria pinnatifida*) to uncooked pasta. The formation of Maillard reaction products (MRP) and other novel antioxidant compounds such as mycosporine-like amino acids during heat treatment of seaweed extracts has been reported [38–40]. Additionally, MRP have proven effective inhibitors of lipid oxidation in cooked minced pork patties [41]. In the present study, MRP formed during heating of L/F_{300} most likely enhanced the DPPH free radical scavenging of cooked minced LTL.

2.5. DPPH Free Radical Scavenging Activity of Seaweed Polysaccharides in Pork Meat Following in Vitro Digestion

During the digestion procedure, cooked minced LTL from each treatment was subjected to pH changes and enzymatic reactions which resulted in increased (~30%–44%) DPPH free radical scavenging activities in digestates compared to undigested aqueous fractions (data not shown). The DPPH free radical scavenging activity of the control post digestion increased from 14.4% to 44.8% and was attributed to the presence of compounds such as peptides released from the pork meat during the *in vitro* digestion procedure. Escudero *et al.* [42] reported 51 different peptides were released from pork meat (*longissimus dorsi*) following *in vitro* digestion. Additionally, peptides obtained from animal sources such as porcine myofibrillar proteins have demonstrated DPPH free radical scavenging activity [43–45]. Data from each treatment (L_{100}, F_{100}, L/F_{100} and L/F_{300}) were adjusted for the meat control to estimate the antioxidant activity due to the seaweed polysaccharides post digestion (Figure 4).

The DPPH free radical scavenging activity of digested L_{100} and L/F_{100} were similar (Figure 4). Laminarin is resistant to digestion in the upper GIT including acidic and enzymatic hydrolysis [46]. Salyers *et al.* [47] established two different types of enzymes (laminarases and β-glucosidases) were essential to fully degrade laminarin and were only synthesised after 4–6 h of incubation in the presence of the inducer. In the present study, the lack of suitable enzymes to break down laminarin in the *in vitro* digestion model used may explain the lack of enhanced antioxidant activity post digestion.

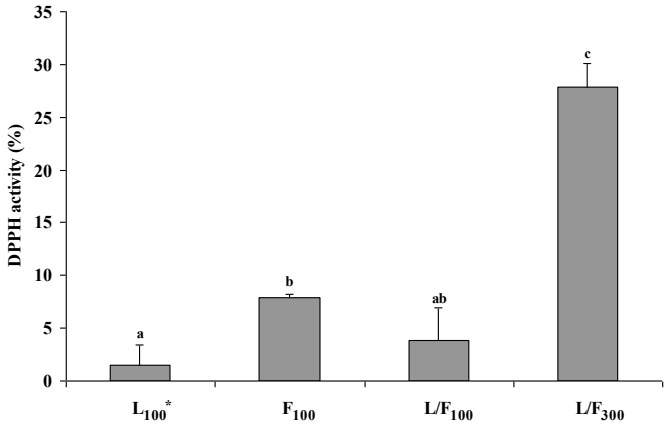

Figure 4. Free radical scavenging activity (DPPH) of L, F or L/F in digested cooked minced *longissimus thoracis et lumborum* (LTL) pork muscle stored for 20 h at ~20 °C. ***** Subscripts 100 and 300 denote concentrations in mg/g. ^{abc} Mean values (± standard deviation error bars) bearing different superscripts are significantly different, $p < 0.05$.

F_{100} and L/F_{300} significantly ($p < 0.05$) enhanced the DPPH free radical scavenging activity of cooked minced LTL post digestion. A few fucan-degrading enzymes have been obtained from marine bacteria and molluscs, however complete enzymatic breakdown has not been reported. The presence of sulphate groups attached to fucoidan has been postulated as a reason for resistance to enzymatic breakdown during digestion. The retention of the sulphate groups during digestion results in high ionic exchange capacities such as the binding of bile salts and scavenging of free radicals throughout the GIT before potential absorption [48]. The enhanced DPPH radical scavenging activity of F_{100} and L/F_{300} in cooked minced LTL, in the present study, may be due to the retention of the sulphate groups throughout the *in vitro* digestion procedure.

The DPPH free radical scavenging activity of digested L/F_{300} was significantly ($p < 0.05$) greater than F_{100}. Fucoidan may be partially responsible for the scavenging activity of the extract. The synergistic effect between components in the L/F extract, such as protein and mannitol, could have contributed to the observed enhanced free radical scavenging activity in cooked minced LTL post digestion. Antioxidant activity, post-digestion, of bioactive peptides extracted from seaweeds has been reported previously [49]. Mannitol is frequently considered as a reference for carbohydrate-type antioxidants due to its established scavenging abilities [24]. Additionally, MRPs formed during cooking may have enhanced the DPPH free radical scavenging activity of L/F_{300} post digestion.

2.6. Bioaccessibility of Seaweed Polysaccharides in Pork Meat after Incubation with Caco-2 Cells

The aqueous fraction of the control and L/F_{300} digestates was incubated with Caco-2 cells for 4 and 20 h to determine the bioaccessibility of L/F post digestion. The DPPH free radical scavenging activity of L/F_{300}, post digestion, was 56.49% higher than the meat control. Following incubation of the control and L/F_{300} digestates with Caco-2 cells for 4 and 20 h, the DPPH free radical scavenging activity of L/F_{300} was 12.34% and 19.85% higher than the meat control, respectively. The reduction in the DPPH free radical scavenging activity indicated theoretical uptake of some compounds with antioxidant activity. Therefore theoretical cellular uptake of seaweed polysaccharides was 44.15% and 36.63% (DPPH free radical scavenging activity) after incubation with Caco-2 cells at 4 and 20 h, respectively. Similarly, Soler-Rivas *et al.* [50] reported a decrease in ABTS free radical activity after digested grilled mushrooms were incubated with Caco-2 cells. Previously reported studies indicated that seaweed polysaccharides can be, to some extent, absorbed into the blood stream post digestion; however metabolism of these components after absorption has not been established [1]. Antioxidant compounds from L/F_{300} not absorbed through the intestinal wall would potentially be available to scavenge free radicals or be fermented by colonic bacteria and contribute to the overall antioxidant defence system of the GIT [1,51]. Further research is necessary to determine the fate of antioxidant compounds after absorption.

3. Experimental Section

3.1. Reagents

All chemicals used were "AnalaR" grade obtained from Sigma-Aldrich Ireland Ltd., Arklow, Co. Wicklow, Ireland and Merck KGaA, Darmstadt, Germany. Tissue culture plastics were supplied by Sarstedt, Wexford, Ireland and the Caco-2 cell line (Human Caucasian colon adenocarcinoma) were

from the European Collection of Animal Cell Cultures, Wiltshire, UK. Fresh pork meat (*longissimus thoracis et lumborum* (LTL)) was supplied by Ballyburden Meat Processors, Ballincollig, Co. Cork, Ireland. Laminarin (L) (MW = 13 kDa) and fucoidan (F) (MW = 57 kDa) standards from Sigma-Aldrich were isolated from *Laminaria digitata* and *Fucus vesiculosus*, respectively. A spray-dried seaweed extract (L/F), containing laminarin and fucoidan was manufactured by Bioatlantis, Tralee, Co. Kerry, Ireland. The extract isolated from brown seaweed (*Laminaria digitata*) was prepared using an acid extraction technique, details of which are industrially-confidential. The extract contained 0.64% protein, 9.3% laminarin, 7.8% fucoidan, and 8.3% mannitol and further details are reported in Moroney *et al.* [15].

3.2. Measurement of the DPPH Free Radical Scavenging Activities of Seaweed Polysaccharides (L, F and L/F)

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity of L, F and L/F was measured using the method of Qwele *et al.* [52] with slight modifications. DPPH (0.2 mM, 3 mL) in methanol was added to 3 mL of L (1 and 10 mg/mL; L_1 and L_{10}), F (1 mg/mL; F_1) and L/F (1 and 3 mg/mL; L/F_1 and L/F_3). Trolox C (1 mg/mL; Trolox), was used as a positive control. Tubes were mixed and incubated for up to 20 h at room temperature (~20 °C) in the dark. The assay control contained 3 mL distilled water and 3 mL of DPPH solution. Absorbance measurements were recorded spectrophotometrically (Cary 300 Bio, UV-Vis spectrophotometer, Varian Instruments, Palo Alto, CA, USA) against a distilled water blank after 1, 4 and 20 h at 517 nm. The DPPH free radical scavenging activity, expressed as a percentage of the assay control was calculated as follows:

$$\% \text{ inhibition of DPPH} = [1 - (\text{absorbance of sample}/\text{absorbance of assay control})] \times 100 \quad (1)$$

3.3. The Effect of Seaweed Polysaccharides on Lipid Oxidation in Pork Muscle Homogenates

Pork homogenates (25% w/v) were prepared by homogenising LTL (70 g) in buffer (210 mL) (0.12 M KCL 5 mM histidine, pH 5.5) on ice using an Ultra-turrax T25 homogeniser. L, F and L/F were solubilised in distilled water and added to LTL homogenates at final concentrations of 3 and 6 mg/mL (L_3, L_6, F_3, F_6, L/F_3 and L/F_6) homogenate. Lipid oxidation in muscle homogenate samples (20 g) held at 4 °C was initiated by the addition of 45 μM $FeCl_3$/sodium ascorbate (1:1). Muscle homogenates with and without $FeCl_3$/sodium ascorbate and without antioxidants (L, F and L/F) were run simultaneously as controls with each experiment. Lipid oxidation measurements were measured after 4 h in samples held at 4 °C.

Measurement of Lipid Oxidation in Pork Muscle Homogenates

A modification of the 2-thiobarbituric acid (TBA) assay of Siu & Draper [53] was used to measure lipid oxidation in pork muscle (LTL) homogenates. Homogenate samples (4 mL) were added to 4 mL 10% trichloroacetic acid (TCA) and centrifuged (Beckman J2-21, Beckman Instruments Inc., Brea, CA, USA) at 6160× *g* for 15 min at 4 °C. Following centrifugation, the supernatant was filtered through Whatman No. 1 filter paper. In a screw cap test tube, the clear filtrate (4 mL) was added to 0.06 M TBA reagent (1 mL) and incubated at 80 °C for 90 min. The absorbance of the resulting coloured complex was measured using a spectrophotometer (Cary 300 Bio) at 532 nm against a blank

containing buffer (2 mL, 0.12 M KCL 5 mM histidine, pH 5.5), 10% TCA (2 mL) and 0.06 M TBA reagent (1 mL). Results were expressed directly as absorbance values at 532 nm.

3.4. The Effect of Seaweed Polysaccharides on Oxymyoglobin Oxidation

3.4.1. Preparation of Commercial Oxymyoglobin

Commercial horse heart oxymyoglobin (OxyMb) was prepared according to a modification of the method of Brown & Mebine [54]. Metmyoglobin (MetMb) (0.06 g) was dissolved in ice-cold distilled water (2 mL) to a concentration of 30 mg/mL and reduced to OxyMb by the addition of sodium dithionite at 1 mg/mL. To remove excess dithionite, OxyMb solution (2 mL) was applied to a glass column (2 cm i.d. × 25 cm) containing 10 g of mixed bed ion exchange resin (Amberlite MB-1A) and eluted from the column with approximately 20 mL cold distilled water. The OxyMb solution was passed through the column three times to reduce the conductivity to that of distilled water and was adjusted to a final volume of 50 mL with double strength buffer (300 mM KH_2PO_4-KOH, pH 5.5). The concentration of OxyMb in the final solution was calculated from its absorbance value at 525 nm divided by a millimolar extinction coefficient of 7.6 $mM^{-1} \cdot cm^{-1}$ [55].

3.4.2. Effect of Seaweed Polysaccharides on Oxymyoglobin Oxidation

Incubates (7 mL) containing OxyMb (~1 mg/mL) and L, F and L/F at two levels (0.1 and 1 mg/mL; $L_{0.1}$, L_1, $F_{0.1}$, F_1, $L/F_{0.1}$ and L/F_1) in 150 mM KH_2PO_4-KOH, pH 5.5, were prepared. Distilled water was used to prepare seaweed polysaccharide solutions (20 mg/mL). Additions to each OxyMb incubate were at a final concentration of 5% (v/v). Incubates were held at 4 °C and OxyMb oxidation was measured on days 0, 4 and 8 of storage.

Following centrifugation at 6160× *g* for 10 min at 4 °C, the absorbance spectra of the incubates (2 mL) containing commercial OxyMb were measured on a spectrophotometer (Cary 300 Bio) and spectral scans were recorded from 750 to 500 nm. The relative proportion of OxyMb (% of total myoglobin) was calculated using absorbance measurements at selected wavelengths (572, 565, 545 and 525 nm) as described by Krzywicki [55].

3.5. Effect of Cooking on DPPH Free Radical Scavenging Activity of Seaweed Polysaccharides in Pork Meat

Fresh minced LTL was assigned to one of five treatments: untreated pork (Control), L (100 mg/g pork; L_{100}), F (100 mg/g; F_{100}), L/F (100 mg/g; L/F_{100}) and L/F (300 mg/g; L/F_{300}). The levels of L, F and L/F added to fresh minced LTL were based on the DPPH free radical scavenging activities of the seaweed polysaccharides determined in Section 2.2. L, F and L/F were dissolved in water, immediately added to fresh minced LTL (5% v/w) and mixed vigorously. Minced LTL (1 g portion) from each treatment was retained for measurement of DPPH free radical scavenging activity of fresh minced LTL prior to cooking. The remaining fresh LTL (5 g portions) of each treatment were placed on aluminium foil lined trays and cooked at 180 °C for 5 min 30 s in a fan-assisted convection oven (Zanussi Professional, Model 10 GN1/1, Conegliano, Italy) until an internal temperature of 72 °C was reached.

Fresh and cooked minced LTL (1 g) were homogenised in 0.05 M phosphate buffer (9 mL), pH 7, using an Ultra Turrax T25 homogeniser and homogenates were centrifuged (Beckman J2-21) at 7800× *g* for 10 min at 4 °C. The supernatant fraction obtained (fresh/cooked minced LTL) was used for the measurement of the DPPH free radical scavenging activity [52]. DPPH (0.2 mM, 3 mL) prepared in methanol was added to 0.3 mL supernatant and 2.7 mL distilled water. The mixture was vortexed and left to stand at room temperature (~20 °C) in the dark. The assay control contained 0.3 mL phosphate buffer and 2.7 mL distilled water and 3 mL of DPPH solution. The absorbance of the solution was measured against a distilled water blank after 1, 4 and 20 h at 517 nm. The scavenging activity of the pork meat against the DPPH radical before and after cooking was expressed as a percentage of the assay control and calculated as:

$$\% \text{ inhibition of DPPH} = [1 - (\text{absorbance of sample/absorbance of assay control})] \times 100 \quad (2)$$

3.6. Effect of in Vitro Digestion on the DPPH Free Radical Scavenging Activity of Seaweed Polysaccharides in Cooked Pork Meat

The *in vitro* digestion procedure was adapted from that previously described by Daly *et al.* [56]. All experimental work was carried out in UV-light free conditions to reduce the possible photo-decomposition of L, F and L/F present in the cooked minced LTL. Briefly, cooked minced LTL (1 g) from each treatment were weighed into 100 mL plastic tubes and homogenized using an Ultra Turrax T25 homogeniser at 24,000 rpm for 10 s in 8 mL Hanks Balance Salts Solution (HBSS) containing BHT. HBSS (5 mL) was slowly pipetted down the homogeniser to rinse remaining residue into the plastic tubes. The homogenates were transferred into amber bottles (rinsed twice using 5 mL HBSS). In order to mimic the gastric phase of digestion, pepsin (1 mL) (0.04 g/mL in 0.1 N HCl) and HBSS (2 mL) was added and the pH was adjusted to 2 using 1 M HCl. Oxygen was displaced by blowing nitrogen over the samples for 5 s. Samples were then incubated at 37 °C for 1 h in an orbital shaking (95 rpm) water bath (Grant OLS200, Keison Products; Essex, UK).

After 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). Subsequently, the pH was increased 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 h. Following intestinal digestion, the digested minced LTL (digestates) from each treatment were centrifuged (Beckman J2-21) at 7800× *g* for 10 min at 4 °C. Undigested minced LTL samples were diluted using HBSS to the same final volume as the digestates and subsequently centrifuged at 7800× *g* for 10 min at 4 °C.

The supernatant (aqueous fractions) of the undigested minced LTL and digestate samples were frozen at −80 °C until required for measurement of DPPH free radical scavenging activity (described in Section 3.5). The assay control contained 0.3 mL HBSS buffer and 2.7 mL distilled water and 3 mL of DPPH solution. The absorbance of the solution was measured against a distilled water blank after 1, 4 and 20 h at 517 nm. The scavenging activity of the pork meat against DPPH radical post digestion was corrected for the meat control and expressed as:

$$\% \text{ inhibition of DPPH} = [(1 - (Ab_{sample}/Ab_{ac})) \times 100] - [(1 - (Ab_{meatcontrol}/Ab_{ac})) \times 100] \quad (3)$$

where Ab_{sample} = absorbance of sample; Ab_{ac} = absorbance of assay control; $Ab_{meatcontrol}$ = absorbance of meat control.

3.7. Bioaccessibility and Theoretical Cellular Uptake of the Aqueous Fraction of Digested Minced LTL

Caco-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), containing 10% (v/v) foetal bovine serum (FBS) and 1% (v/v) non-essential amino acids. Cells were grown at 37 °C /5% CO_2 in a humidified incubator and were cultured with 0.5% Penicillin-Streptomycin (5000 U/mL). Cultures of Caco-2 cells were used between passages 46–51. To establish the Caco-2 intestinal model, the cells were seeded at a density of 6×10^4 cells cm^{-2} on a transwell plate (12-well plate, 22 mm diameter, 0.4 μm pore size membrane). Media was changed every 2–3 days and experiments were performed when monolayers were 17–20 days post-confluency. The aqueous fraction of the digestates (control and L/F_{300}) (125 μL) were diluted to a final volume of 500 μL with serum free media and added to the top chamber of the transwell plate. Serum free media (1 mL) was added to the basolateral chamber and the cells were incubated for 4 and 20 h. Preliminary work showed that the aqueous fraction of the digestates was not toxic to the cells (data not shown). The transepithelial electrical resistance (Millicell-ERS, Millipore, Cork, Ireland) was measured before and after the addition of the aqueous fraction of the digestates to ensure the monolayer remained intact. The media from the basolateral chamber was then harvested for the measurement of the DPPH free radical scavenging activity (see Section 3.5).

The assay control contained 0.3 mL serum free media and 2.7 mL distilled water and 3 mL of DPPH solution. The absorbance of the solution was measured against a distilled water blank after 4 h at 517 nm. The difference between the DPPH free radical scavenging activities of L/F_{300} and the control, expressed as a percentage of the control, was calculated for the aqueous fraction of the digestate (AF) and the transwell basolateral chamber media (TW) as follows:

$$\begin{gathered}\%\ \text{theoretical cellular uptake of antioxidant compounds} = \\ [(AF_{L/F300} - AF_{meatcontrol})/AF_{meatcontrol}) \times 100] - [(TW_{L/F300} - TW_{meatcontrol})/TW_{meatcontrol}) \times 100]\end{gathered} \quad (4)$$

where $AF_{L/F300}$ = absorbance of aqueous fraction of the digestate L/F_{300}; $AF_{meatcontrol}$ = absorbance of aqueous fraction of the digestate meat control; $TW_{L/F300}$ = absorbance of transwell basolateral chamber media following incubation of L/F_{300} with Caco-2 cells; $TW_{meatcontrol}$ = absorbance of transwell basolateral chamber media following incubation of the meat control with Caco-2 cells. The difference in activity between AF and TW was attributed to theoretical uptake of antioxidant compounds by the Caco-2 cells.

3.8. Statistical Analysis

Each experiment was carried out three individual times. All analyses were performed in duplicate. The DPPH free radical scavenging activities of L, F and L/F, fresh and cooked LTL pork muscle, cooked LTL digestates and lipid oxidation mean values were analysed by one-way ANOVA. Means were considered significantly different at ($p < 0.05$) using Tukey's post hoc test. A full repeated measures ANOVA was conducted to investigate the effects of L, F and L/F concentration and time on oxymyoglobin oxidation. L, F and L/F represented the "between-subjects" factor and the effect of time

was measured using the "within-subjects" factor. Tukey's test was used to adjust for multiple comparisons between treatment means ($p < 0.05$). All analysis was carried out using the SPSS 18.0 for Windows (SPSS, Chicago, IL, USA) software package.

4. Conclusions

Due to the presence of sulphate groups and anionic charge, fucoidan is a more potent free radical scavenging antioxidant than laminarin. Furthermore fucoidan is at least, in part, responsible for the antioxidant activity observed by the L/F extract in previous studies. Fucoidan may be a potential natural antioxidant to enhance lipid stability in meat products. The antioxidant potential of fucoidan and the L/F extract is strongly influenced by the cooking and digestion processes. The L/F extract demonstrated superior antioxidant activity compared to fucoidan in minced LTL, after cooking and post digestion. The antioxidant compounds of the L/F extract were partially absorbed by Caco-2 cells confirming their bioaccessibility post digestion. Results demonstrate the potential for extracts containing fucoidan to enhance antioxidant activity of functional cooked meat products as well as contribute to human antioxidant defence systems.

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Author Contributions

Experiments were designed by N.M., M.O'G., S.L., C.S. and J.K., and conducted by N.M. and S.L. (cell culture work). All authors contributed to the analysis and interpretation of experimental data and the writing and review of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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