Black soldier fly chitosan characterisation for food contact applications.

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy.

Sean Liam Mason

School of Natural and Environmental Sciences

Newcastle University

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Abstract

Plastic pollution and food waste contribute to detrimental impacts. Many foods are wasted because they spoil rapidly. Plastics are a packaging material for many foods. Minimising food waste and plastic usage is necessary in combination with increased sustainable food production to provide food security. Biopolymer-rich waste materials are viable sources of packaging alternatives. Insect bioconversion is studied for securing future protein supply. Chitin is abundant in waste material. Chitin's deacetylated derivative, chitosan, has properties that remain poorly characterised towards antimicrobial applications. Therefore chitosan was extracted and characterised from *Hermetia illucens*, antimicrobial properties were investigated and impacts on coated foods monitored. Experimental evidence is presented which contributes to chitosan characteristic-dependent antimicrobial action and exhibits the effects of chitosans as a coating material.

Chitosans were prepared using chemical methods and characterised by degree of acetylation and molar mass. Antimicrobial susceptibility tests were performed against foodborne bacteria and chitosans with different properties were screened against *Bacillus* reporter strains to study mode of action. Chitosans showed broad-spectrum antimicrobial activity. *Bacillus* reporters highlighted a characteristic-dependent effect in which chitosans with larger molar masses and high degrees of deacetylation induced positive responses for a fatty acid synthesis inhibition reporter, while the lower molar mass, less deacetylated chitosan had no positive reporter responses. Subsequently, chitosans were applied as coatings and a layer-by-layer assembly method was assessed in combination with alginate. Spoilage characteristics were monitored over time. Chitosan coating had a significant impact on microbial load in poultry meat. A layer-by-layer assembly method showed lower colony counts than chitosan alone, which could have a significant impact on poultry meat spoilage.

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List of Abbreviations

^{13}C NMR	Carbon-13 nuclear magnetic resonance
^{1}H NMR	Proton nuclear magnetic resonance
ACC	Aerobic colony count
ADF	Acid detergent fibre
ADL	Acid detergent lignin
AF4	Asymmetric flow field flow fractionation
AmmAc	Ammonium acetate
AST	Antimicrobial susceptibility test
BSF	Black soldier fly
BSFP	Black soldier fly prepupae
CDA	Chitin deacetylase
cps	Centipoise
CS	Chitosan
D_2O	Deuterium oxide
DA	Degree of acetylation
DCl	Deuterium chloride
DDA	Degree of deacetylation
ddH_2O	Deionised water
DES	Deep eutectic solvents
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FEP	Fluorinated ethylene propylene
FTIR	Fourier-transform infrared spectroscopy

GAG	Glycosaminoglycans
GC-MS	Gas Chromatography Mass Spectrometry
GlcNAc	*N*-acetyl-ᴅ-glucosamine
GPC	Gel permeation chromatography
GRAS	Generally recognised as safe
HACCP	Hazard analysis and critical control point
HAc	Acetic acid
HCl	Hydrochloric acid
HG	Homogalacturonan
HMW	High molecular weight
IgE	Allergen-specific immunoglobulin E
IL	Ionic liquids
IPTG	Isopropyl β-ᴅ-1-thiogalactopyranoside
KBr	Potassium bromide
LBL	Layer-by-layer
LPG	Lysyl phosphatidylglycerol
LPMO	Lytic polysaccharide monooxygenase
LPS	Lipopolysaccharides
LS	Light scattering
MALS	Multi angle light scattering
MDA	Malondialdehyde
MIC	Minimum inhibitory concentration
MM	Molar mass
MMW	Medium molecular weight

MRD	Maximum recovery diluent
MW	Molecular weight
NaAc	Sodium acetate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NBULMW	New batch ultra low molecular weight
PCR	Polymerase chain reaction
PG	Peptidoglycan
Ppb	Parts per billion
Ppm	Parts per million
Rg	Radius of gyration
RI	Refractive index
RNA	Ribonucleic acid
SDGs	Sustainable development goals
SEC	Size exclusion chromatography
SLS	Static light scattering
STEC	Shiga toxin-producing *Escherichia coli*
TBARS	Thiobarbituric acid reactive substances
TSP	Trimethylsilylpropanoic acid
TVC	Total viable count
ULMW	Ultra low molecular weight
UN	United Nations
VLMW	Very low molecular weight
VTEC	Verotoxigenic *Escherichia coli*

X-Gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

Chapter 1. Introduction

1.1 Three crises on Earth: climate change, nature/biodiversity loss and waste and pollution.

In the last 150 years the global population has risen dramatically. Seventy years ago the population was estimated at 2.6 billion, it has nearly tripled since then to 7.7 billion and it is anticipated to climb higher by the end of the century (11 billion) (UN, 2021a). Human activities have significant impacts on the planet and on other living organisms. For example, the use of fossil fuels to provide fuel and materials has resulted in contamination of the biosphere in gaseous and micro solid forms (UN, 2019). Greenhouse gases produced from burning fossil fuels continue to rise to unprecedented levels (UN, 2021d). This is leading to atmospheric temperature increases, which in turn are causing detrimental environmental effects. The rising temperatures are melting the ice caps, leading to more water in our oceans, meaning that sea levels will rise (UN, 2021d).

90% of natural disasters are weather-related, including floods and droughts (WFP, 2021a; WFP, 2021b). These are becoming more common and cause fatalities as well as crop destruction. Examples include floods and landslides in 2020 in East Africa, the Middle East, and South Asia (WFP, 2021a; WFP, 2021b), Cyclone Eloise in Mozambique and South Africa in 2021 (WFP, 2021a; WFP, 2021b), and tropical storms, hurricanes and flooding in Central America, areas where prolonged droughts are more common (WFP, 2021a; WFP, 2021b).

Pollution caused by human activities is contaminating environments and resources needed for the increasing population. It is claimed that we have lost a third of arable land in the past 40 years due to pollution (Milman, 2015). Water pollution resulting in eutrophication can eventually result in a reduction in biodiversity. We have introduced invasive species and harvested resources to extremes, ultimately causing species extinctions and reductions in biodiversity.

Plastics produced from fossil fuels have been found everywhere on Earth even in places that humans cannot inhabit such as the Mariana trench (Gibbens, 2019; Morelle, 2019). They can take a long time to degrade, eventually breaking down into microparticles that are not able to be removed during water treatment processing (NationalGeographic, 2021; UNEP, 2021b). They can bind with toxins and present a serious health risk when consumed (NationalGeographic, 2021; UoB, 2021). They have been found in various organisms in particular in marine habitats and end up bioaccumulating through the food chain

(NationalGeographic, 2021; UoB, 2021). They have been found in human excrement and in tap water, as well as meats in supermarkets (NationalGeographic, 2021; UoB, 2021).

As the population increases, these detrimental environmental impacts put further strains on human populations, pushing more people into challenging situations. Water is not available to many despite being a human right. The agricultural industry is responsible for 70% of water use (UN, 2021c), whilst 2.2 billion people lack safe drinking water and 2 billion people live in countries experiencing high water stress (UN, 2021c).

Food crises are also a concern. These can be a result of food not being readily available, or due to affordability. Three billion people are unable to afford a healthy diet (WFP, 2021a; WFP, 2021b) and in 2019 690 million were affected by hunger (WFP, 2021a; WFP, 2021b). As Covid-19 effects hit, this is expected to rise even further. Millions of people were left with job losses and lower income, and coupled with increased food costs, left some people unable to afford to eat properly (WFP, 2021a; WFP, 2021b).

This unsustainable production and consumption led to the UN introducing targets for reducing our impact on the environment with the 17 Sustainable Development Goals (SDGs). The UNs first and second SDGs are related directly to human suffering from lack of food and water. There is an increased need to reduce extreme poverty and improve food security globally. The population increase correlates with an increased global demand for protein production in a sustainable manner. It is estimated that 50% more food is required by 2050 (Milman, 2015). However, producing enough food and providing sufficient water for the increasing population is becoming a significant challenge.

Despite this need for food, food waste is high. The UN states that 33% of the world's food is wasted and still over 800 million people are malnourished (UNEP, 2021a). Food waste also contributes to greenhouse gas emissions, requires waste management systems, increases food insecurity, and is considered a major contributor to the three planetary crises: climate change, biodiversity loss and waste and pollution (UNEP, 2021a). The UN aims to halve food waste by 2030 (UN, 2021b). The UK government aims to reduce food waste by 20% by 2025 (DEFRA, 2020). In 2019, 931 million tonnes of food was wasted (UNEP, 2021a). It has also been noted that this food wastage is happening in high and low income countries. Many of the food groups highlighted in the UK and Japan by Parry, Bleazard and Okawa (2015) are wasted because they have not been consumed fast enough before decaying to unsafe levels. For example when compared by cost, Parry, Bleazard and Okawa (2015) evidenced that meat

and fish contributed the highest cost to food waste. They also highlighted that fresh vegetables and salads contributed the most to food waste when compared by mass. When reasoning for the food waste was considered fresh vegetables and salads, as well as fruits and baked goods had the highest proportions of avoidable food waste due to not being used before spoilage takes effect.

Plastics are most commonly used to contain food products and increase shelf life helping to reduce food waste, but contribute to the contamination of the environment. Single use plastics are a significant concern as they are not recycled and usually end up in landfill waste, eventually further polluting the environment (NationalGeographic, 2021). Therefore, there is a significant amount of interest in developing effective but sustainable ways to produce alternatives to plastics, to produce protein for the population and preserve foods for longer periods of time. For example in the UK, businesses have been offered £1.15 million to invent ways that help minimise food waste (DEFRA, 2020). Plastics are polymers which are large chains composed of smaller individual units. As an alternative to harmful plastics, biologically produced polymers are studied for their potential to replace these harmful polymers.

1.2 Polymers focussing on natural polyelectrolyte polymers

Polymers are large molecules composed of many individual components (monomers) bonded together. They are incredibly useful for a range of applications and are abundant in nature. Due to the different moieties found on the individual components of polymers, they can function and be categorised differently. Humans have also synthesised their own polymers from, for example, crude oil fractions (e.g. polypropylene). Examples of different polymers include: epoxies, plastics, fibres and polyelectrolytes that can be natural (biopolymers) or synthetic.

Polymers can be found in all phyla. Their structural complexity relates to their natural function. Currently, the most well-known biopolymers are derived from plants, fungi and animals. One of the biggest challenges for biopolymers is the need to meet the same (or better) performance standards as the synthetic polymers they are to replace. Several biopolymers have been reported to have poor mechanical properties, which limit their applications. Still, the biopolymer market is forecast to increase massively in the next decade (Research, 2017). The market for biodegradable polymers is vast and is expanding into billions of US dollars (USD). The market is forecast to be worth over 6.5 billion USD in 2023 and has an estimated compound annual growth rate over 10% for the next five years (Research, 2017). The global market for chitosan is regularly measured in billions of US dollars and with compound annual growth rates above 15% for the next five years (Insights, 2018; Research, 2019).

Some popular biopolymers are fairly recognisable due to advertising when they exhibit unique bioactivities. Hyaluronic acid and collagen are frequently mentioned in cosmetic advertisements.

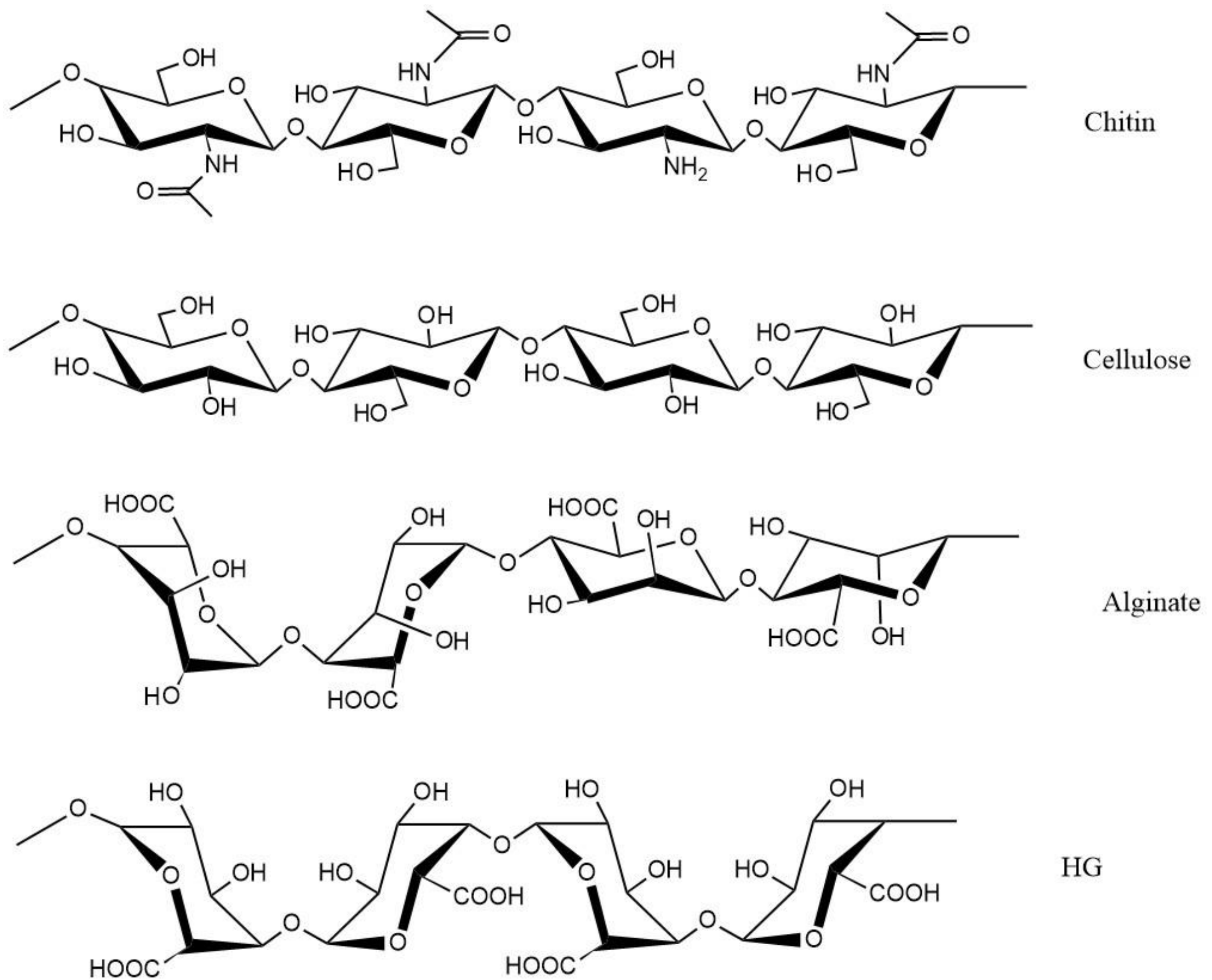


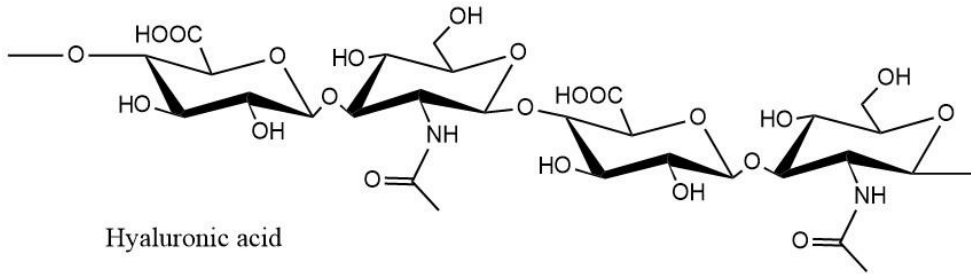
Figure 1 – Structures of chitin with 75% acetylation composing of three *N*-acetyl-ᴅ-glucosamine and one ᴅ-glucosamine residues, (D) β-(1→4) linked, cellulose, β-(1→4) linked ᴅ-glucose units, alginate with two α-ʟ-guluronic (G) and two β-ᴅ-mannuronic acid (M) linked (1→4) and homogalacturonan (HG) (linear α-(1→4)-linked galacturonic acid).

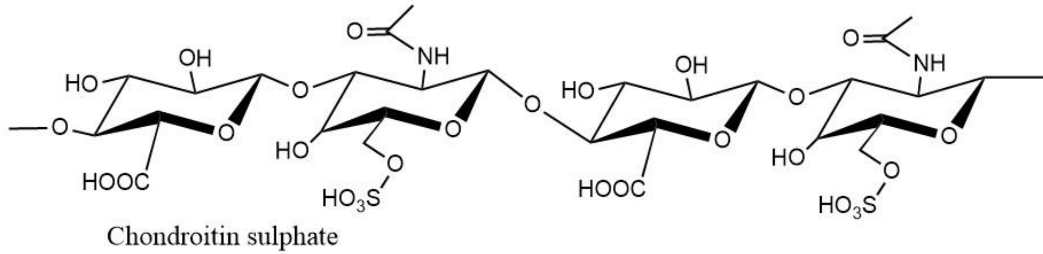
One of the most structurally and functionally diverse categories of biopolymers is the glycans, the structures of some common polysaccharides are included in Figure 1. In the plant kingdom, glycans compose significant components of the cell wall for structure and signalling. Cellulose, β-(1→4) linked ᴅ-glucose units, is the most abundant polysaccharide in nature and provides structure to the plant cell wall. This has been exploited as a biological polymer and provides a platform chemical for other polymer derivatives that increase its functionality such as nanocellulose, cellulose nanocrystals, and carboxymethyl cellulose. Furthermore, it can be hydrolysed to produce other renewable chemicals such as 5-hydroxymethyl furfural, which can be transformed into dimethylfuran, which shows promise as a biofuel (Román-Leshkov *et al.*, 2007).

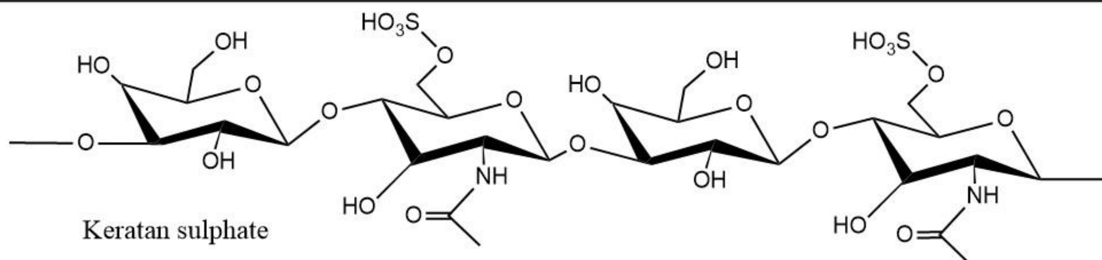
Pectins are more complex polysaccharides that are rich in galacturonic acid (GalA) residues. These connect with rhamnogalacturonans (RG1 and RG2) which are composed of rhamnose, galactose and arabinose (Fry, 2004). A significant portion of pectin is composed of homogalacturonan (HG) (linear α-(1→4) -linked GalA homopolymer), which is esterified in the plant cell wall. Specific enzymes, pectin methylesterases, can then alter the arrangement of ester groups on the pectin chains which adds variation to the structure and allows interactions with oppositely charged molecules (Willats *et al.*, 2001).

Another interesting plant derived uronic acid based polysaccharide can be isolated from brown algae. Alginate, comprised of different ratios of β-ᴅ-mannuronic acid (M) and α-ʟ-guluronic acid (G) linked (1→4), has also received some media attention for its appeal in making edible water bubbles through its calcium based gelation process (Grant *et al.*, 1973). Brown algae constitute a significant segment of biomass in littoral zones therefore the biopolymer is in considerable abundance and particularly attractive as it is not using land that would otherwise be used for food. Alginate also has biotechnological uses in a range of areas including biomedicinal applications (Lee and Mooney, 2012).

In the animal kingdom, glycans take on a variety of complex roles and have also been developed for biotechnological purposes. In mammals, most notably the glycosaminoglycans (GAGs) have elaborate anionic structures. They are composed of repeating units consisting of a uronic and an amino saccharide. They are of significant commercial interest for their biomedical applications including anti-inflammatory, antioxidant, immunomodulatory and neuroprotective properties (Balbinot-Alfaro *et al.*, 2021). GAGs are categorised into six forms, five of which are sulphated. The five sulphated GAGs are chondroitin sulphate, dermatan sulphate, keratan sulphate, heparan sulphate, and heparin (Balbinot-Alfaro *et al.*, 2021). These five categories of glycan are extremely structurally complex as they have multiple molecular units, which vary significantly. Figure 2 indicates the basic structures. The only non-sulphated GAG is hyaluronic acid which is present in the extracellular matrices of mammals. Its repeating disaccharide is composed of ᴅ-glucuronic acid and *N*-acetyl-ᴅ-glucosamine, linked via alternating β-(1→4) and β-(1→3) glycosidic bonds (Balbinot-Alfaro *et al.*, 2021). The anionic moieties present mean they behave as polyelectrolytes in solution.


Hyaluronic acid


Chondroitin sulphate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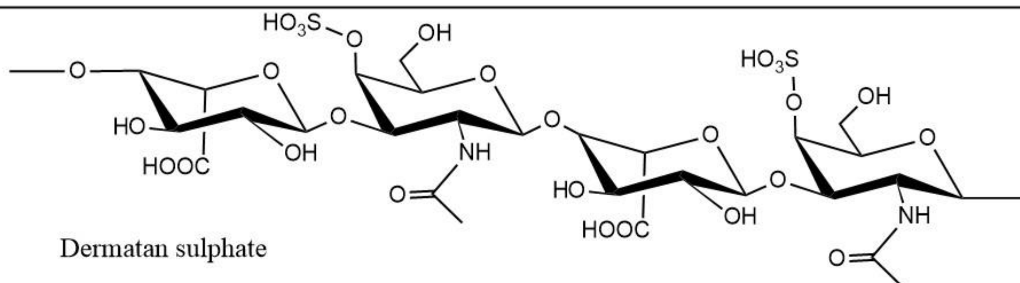
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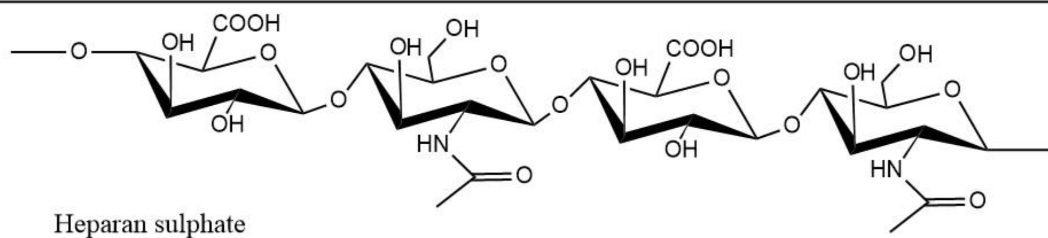
Heparan sulphate

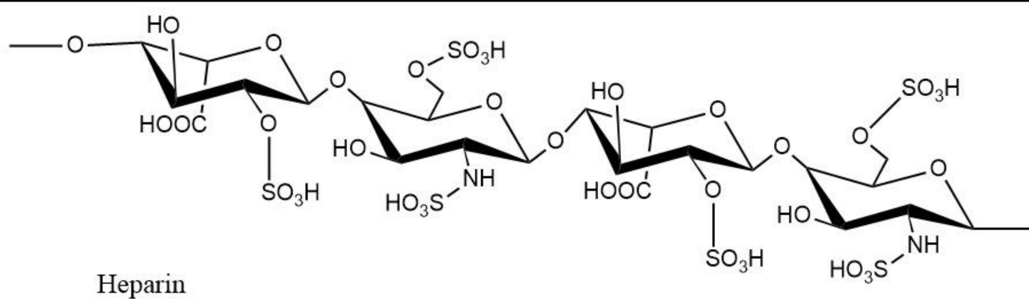
Heparin

Figure 2 – The basic structures of the six glycosaminoglycans (GAGs) (Balbinot-Alfaro *et al.*, 2021). Hyaluronic acid (ᴅ-glucuronic acid and *N*-acetyl-ᴅ-glucosamine), chondroitin sulphate (β-*N*-acetyl-d-galactosamine and β-glucuronic acid), keratan sulphate (*N*-acetylglucosamine and galactose), dermatan sulphate (*N*-acetyl-galactosamine and iduronic acid), heparan sulphate (glucuronic acid linked to *N*-acetylglucosamine) and heparin (iduronic acid and glucosamine, sulphated variably).

Polyelectrolytes are polymers with a substantial portion of ionisable moieties providing anionic, cationic or amphiphilic properties (Lalevée *et al.*, 2016). They can provide important functions for applications in areas such as gelling agents in food and excipients of active molecules. Polyelectrolytes have been synthesised, examples include polylysine and polyethylene imine. Polyelectrolytes also occur abundantly in nature including DNA/RNA, proteins, glycans, and polyamines. Polyelectrolyte functional applications can come from the electrostatic interactions with oppositely charged molecules which allow the formation of complexes. These can then provide unique formations such as colloidal suspensions, biphasic systems and multilayers (Lalevée *et al.*, 2016).

1.3 Chitin

Chitin was first described in fungi by Henri Braconnot in 1811 (Braconnot, 1811). Chitin is found in many taxa including arthropods, molluscs, fungi, protists, sponges, some bacteria and algae (Merzendorfer, 2011). It is the second most abundant polymer on Earth after cellulose. In arthropods, chitin is the main polysaccharide present in the cuticle (Zhu *et al.*, 2016). The largest source of chitin comes from shellfish waste. Shellfish waste amounted to 6–8 million tonnes annually in 2015 (Yan and Chen, 2015). Chitin production was estimated at 28000 tonnes in 2015 (SFly, 2019). The global market for chitin and its derivatives was estimated at 63 billion USD in 2015 (Yang and Yan, 2018).

Chitin is composed of *N*-acetyl-ᴅ-glucosamine sugars linked with β-(1→4) linked glycosidic bonds, and thus differs from cellulose by the addition of an acetyl amine group on the second carbon atom in the glucose ring, as depicted in figure 1. There are therefore a lot of similarities in the research conducted on these two polysaccharides. However, the amine group in chitin is more commercially appealing as it can be used as a precursor for more chemical derivatives.

In nature chitin is found associated with different compounds (Zhu *et al.*, 2016). To be utilised for applications it needs to undergo purification treatments. These have conventionally included the use of acid and alkali treatments (Liu *et al.*, 2012), but there is no standard method of extraction, nor is the method specific to any particular chitinous material. In addition, different extraction methods are being developed to improve the sustainability of the process. These include enzyme based methods, such as fermentation and the use of proteolytic enzymes (Caligiani *et al.*, 2018; Younes and Rinaudo, 2015), and green chemistry based methods using deep eutectic solvents and ionic liquids (Sharma *et al.*, 2013).

Chitin has been isolated from a range of species and varies depending on its biological source. However, this interspecies variability may also be associated with incomplete isolation procedures. It has two main structural isoforms, alpha, and beta, which are defined by the alignment pattern of chitin chains (Roberts, 1992). These result in differential hydrogen bonding and different reactivity. Alpha chitin is the most abundant and is most commonly isolated from the exoskeleton of arthropods. It is extensively hydrogen bonded and crystalline making it highly insoluble (Roberts, 1992). Chitin's solubility is limited to strong chemicals such as lithium chloride/dimethyl acetamide solutions (Rinaudo, 2006).

However, green chemistry methods such as ionic liquids and deep eutectic solvents are showing promise (Sharma *et al.*, 2013; Qin *et al.*, 2010). Beta chitin is less abundant but reportedly more reactive. It can be extracted from the internal pen of cephalopod molluscs (Rinaudo, 2006).

Chitin can be modified to provide polymers with more functionality. Modifications to chitin include its deacetylation, acetylation, maleylation, phthaloylation and napthaloylation (Ifuku, 2014; Ifuku and Saimoto, 2012). Surface deacetylated chitin nanofibers, nanowhiskers and nanocrystals are also of significant interest (Ifuku, 2014; Ifuku and Saimoto, 2012).

Chitin can be hydrolysed to produce oligosaccharides, disaccharide and monosaccharide units. Lower molecular weights have increased solubility which can help in many applications. The depolymerised chitin can be chemically transformed into other useful chemicals such as 3-acetamido-5-acetylfuran (3A5AF), 5-hydroxymethylfurfural (5HMF – like cellulose) and the pyrazine fructosazine (Shamshina and Berton, 2020). 3A5AF is promising for multistep syntheses of bioactive chemicals including proximicin and aminofuran antibiotics (Shamshina and Berton, 2020). 5HMF is also derived from cellulose, it can be transformed into a candidate for biofuel (van Putten *et al.*, 2013). It can produce levulinic acid, 5-ethoxymethylfurfural, and furan-2,5-dicarbaldehyde (van Putten *et al.*, 2013). Fructosazine has been reportedly used for treatment of osteoarthritis. It can also be used for flavouring agents and fragrances (Shamshina and Berton, 2020; Yang and Yan, 2018).

1.4 Chitosan

The most well-known derivatives of chitin are chitosans, discovered by Charles Rouget in 1859 by boiling chitin in potassium hydroxide (Rouget, 1859). Terminology of chitosan can often be confused. 'Chitosan' can be used to identify a molecule, but it generally refers to a family of molecules that behave differently depending on the acetylation and polymerisation characteristics. These are the deacetylated derivatives of chitin, which are linear copolymers consisting of *N*-acetyl-D-glucosamine and D-glucosamine units that can be produced with different degrees of deacetylation (DDA) and molecular weights (MW). This results in different properties such as solubility, antimicrobial and antioxidant activities (Aranaz *et al.*, 2009). Chitosan can also be the source of further derivatives due to the more easily accessible amine group's nucleophilic nature.

Deacetylation is most commonly achieved through treatment with concentrated sodium hydroxide at high temperatures. However, similar to the ester groups of pectin, there exist enzymes that can perform this action called chitin deacetylases (CDAs) (Hoell, Vaaje-Kolstad and Eijsink, 2010). Chitin deacetylases remove the acetyl groups from chitin, although similar to several other chitin active enzymes, their function appears to be limited to amorphous regions (Jaworska and Roberts, 2016).

The deacetylated units, D-glucosamine, have free amino groups which become cationic in acidic media and improve solubility. Chitosan dissolves in weak acid and has a pKa in the range of 6.0-6.5 (Strand *et al.*, 2001). It also precipitates in strong acid (>1 M HCl) (Rinaudo, 2006). Chitosan in solution therefore behaves as a weak base, a cationic polyelectrolyte. The dissolution of chitosan has also been reported at neutral pH in the degree of acetylation (DA) range of 0.4-0.7% (Rinaudo, 2006). However, this is usually achieved through reacetylation of chitosan with acetic anhydride (Rinaudo, 2006), rather than directly from the deacetylation of chitin.

Chitosan is differentiated from chitin by the proportion of acetyl groups remaining on the chain and can be easily differentiated from chitin polymer in the laboratory by its solubility in 1% (v/v) acetic acid. When the chains are composed of more D-glucosamine units than *N*-acetyl-D-glucosamine residues it is typically referred to as chitosan. The composition of residues can be measured by a range of methods including ^{1}H NMR (Hirai, Odani and Nakajima, 1991; Lavertu *et al.*, 2003; Vårum *et al.*, 1991a), ^{13}C NMR (Vårum *et al.*, 1991b) and Fourier-transform infrared spectroscopy (FTIR) (Brugnerotto *et al.*, 2001a). The proportion of

each residue on the chain is referred to as the degree or fraction of acetylation which is an average measurement for the polymer sample.

The pattern with which the acetyl groups remain on the chain remain a topic of discussion due to the way other polysaccharides with additional functional groups have been characterised. Most notable are pectic HG and its degree of esterification, and alginate's ratio of its different uronic acid residues. However, no significant difference in distributions have been evidenced for chitosans, even between different deacetylation techniques (Thevarajah *et al.*, 2017; Vårum *et al.*, 1991a; Vårum *et al.*, 1991b). Novel methods for studying the pattern of acetylation in chitosan polymers are highly sought after.

Chitosan is also characterised by its molecular weight (MW). This has a significant effect on its properties such as solubility and bioactivity, as low molecular weights are often soluble regardless of the degree of acetylation. Molecular weight is most commonly measured through a combination of gel permeation chromatography (GPC) coupled with a viscometer and light scattering (Brugnerotto *et al.*, 2001b). This provides an average MW measurement, along with the MW distribution of the sample. However this is expensive equipment, and as chitosan research continues to expand, alternative lower cost options include different types of viscometric measurements. While sample polydispersity can mathematically be calculated from capillary viscometry (Kulicke and Clasen, 2004), the viscometric methods do not directly measure differences in MW distribution.

The free amine group that chitosan possesses is the primary source of its significant research interest, properties and therefore many of its applications. This also permits a number of different derivatives to be produced. These include quaternary ammonium salts to increase the charge density, *N*-succinyl chitosan that provides an anionic charged polymer, and carboxymethyl chitosan and a range of Schiff base derivatives. Schiff base derivatives are synthesised from reactions of the amino group with an active carbonyl, ketone or aldehyde (Barbosa *et al.*, 2019), as depicted in Figure 3.

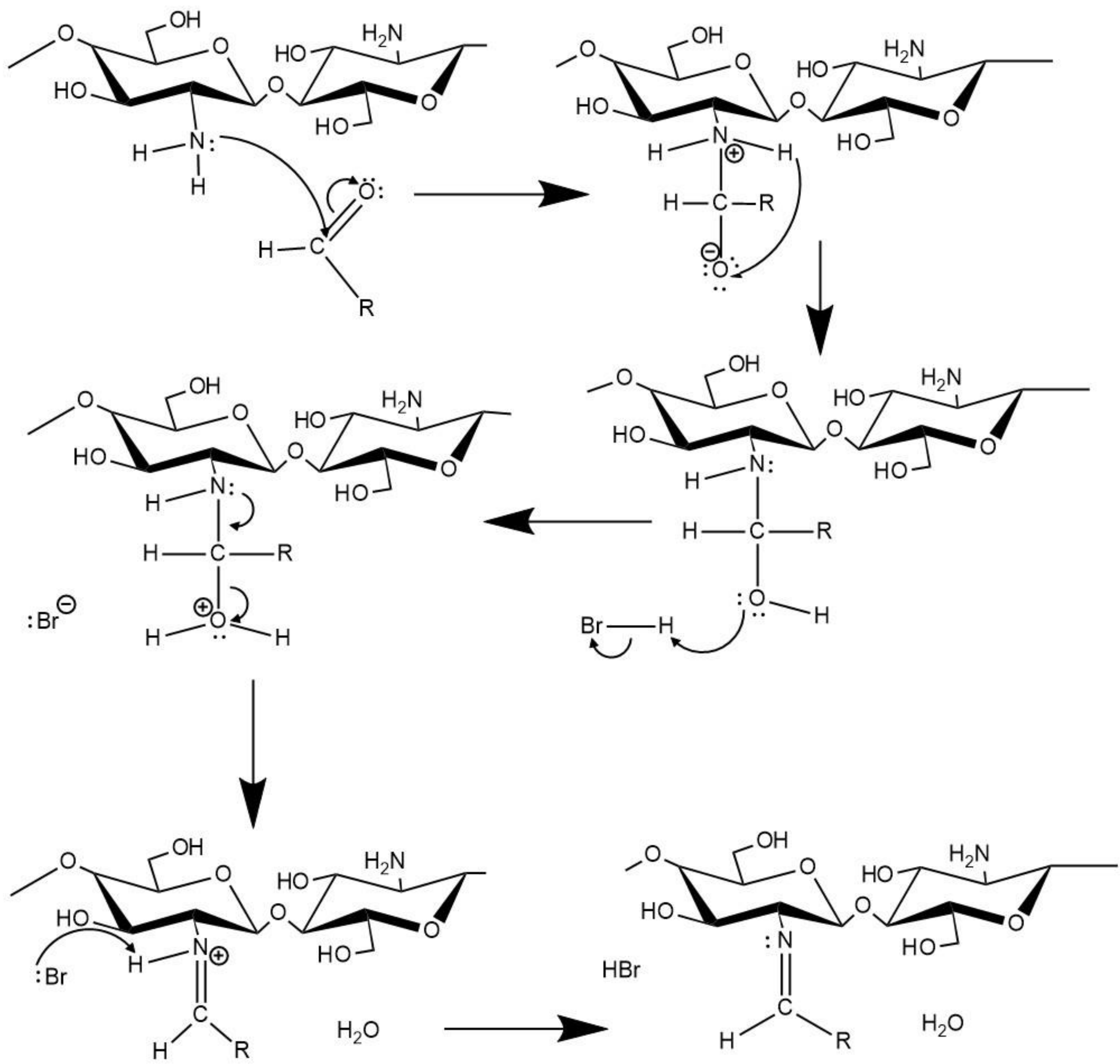

Figure 3 – The nucleophilic addition mechanism for the formation of Schiff base derivatives of chitosan. Dots indicate free electron pairs and curly arrows highlight the movement of electrons to enable the formation of new bonds.

1.5 Properties of chitosan

Chitosan's polyelectrolyte behaviour largely contributes to its many properties for different applications, emphasising the importance of characterising the average degree of acetylation and molecular weight of the sample. Chitosan has functions for mucoadhesion, antimicrobial, haemostasis, antioxidant, biocompatibility and permeation enhancement among others.

For mucoadhesion, chitosan's cationic charges are complementary to the anions present in the sialic acid residues of the protein mucin (Illum *et al.*, 2001). Chitosan can also facilitate haemostasis because the surface of erythrocyte membranes are negatively charged meaning they can be attracted to positively charged chitosan causing aggregations (Baldrick, 2010). As a permeation enhancer chitosan can interact with negatively charged structures on the cell surface membrane, facilitating transport of hydrophilic molecules across the cell surface membrane by moving proteins in tight epithelial junctions (Artursson *et al.*, 1994). Chitosan also selectively interacts more with certain cells, making certain treatments more effective (Erman and Veranič, 2018).

Chitosan is known to be biocompatible due to low immunogenicity and cytotoxicity (Aranaz *et al.*, 2009; Konovalova *et al.*, 2017). In addition, as a naturally produced polymer, enzymes have evolved to be able to degrade chitosan. Many enzymes whose primary function is not chitosan degradation also possess chitosan degradation capabilities. In fact it has been stated that when categorising cellulases and chitosanases there is regularly cross compatibilities (Hoell, Vaaje-Kolstad and Eijsink, 2010). Large scale production of chitosan and its implications for microbial communities in the environment are yet to be fully investigated. This could be a significant milestone in the production and use of chitosan in its many applications. Furthermore chitosan has mild antioxidant properties in which it is able to scavenge free radicals and chelate metal ions associated with food spoilage (Aranaz *et al.*, 2009).

Finally, the antimicrobial property plays a significant part in many of chitosan's applications. Chitosan exhibits antimicrobial properties against gram positive and negative bacteria, and some fungi (Kong *et al.*, 2010; Raafat *et al.*, 2008; Rabea *et al.*, 2003; Verlee, Mincke and Stevens, 2017; Younes *et al.*, 2014). Several mechanisms of action have been proposed but not fully confirmed (Verlee, Mincke and Stevens, 2017). The variations in structure, due to differences in molecular weights (ranges and methods used) and degree and distribution of

acetyl groups, as well as the absence of important sample preparation details make reproducibility challenging. It is speculated that the variability of these properties can induce different antimicrobial activities (Verlee, Mincke and Stevens, 2017). For example lower molecular weight chitosans are suggested to enter the cell and disrupt DNA and RNA synthesis, while higher molecular weights are speculated to disrupt negatively charged structures on cell surface membranes resulting in leakage of intracellular material (Verlee, Mincke and Stevens, 2017). Chitosans with a lower degree of acetylation tend to have the best antimicrobial activity, which is associated with the charge density of the cationic free amino groups. A large amount of research focusses on modifying chitosan to produce more active molecules due to this feature, including increasing the number of amine groups (quaternary amines) and other Schiff base derivatives (Barbosa *et al.*, 2019; Varlamov *et al.*, 2020). Molecular weight effects are difficult to compare in a fair manner due to the technical difficulties that viscosity can introduce to antimicrobial susceptibility methods. Molecular weight limits the potential mechanisms of antimicrobial action as larger molecules will not be able to enter into cells. Therefore, the molecular weight distribution and the variation in the average degree of acetylation of samples are important during experiments attempting to elucidate different activities associated with structural characteristics.

Chitosan’s properties enable it to span a wide array of different applications. With applications offering solutions to some significant challenges in biomedical science, food, agriculture and cosmetics.

1.6 Chitosan Applications

1.6.1 Biomedical

Chitosan's biomedical applications can be broken down into three main areas: tissue scaffold, pharmaceutical excipient and wound treatment applications. Chitosan's biocompatibility is an important property in biomedical applications. Its antimicrobial properties can also be considered an important feature for tissue scaffolds and wound healing applications. A chitosan based wound dressing received approval for medical use in the US in 2003 (Zhang, Gao and Liu, 2015).

In wound healing applications chitosan can be used for its haemostatic properties (Baldrick, 2010). Its polyelectrolyte complexes also help in this regard. It has been applied for burns and cutaneous ulcers (Boucard *et al.*, 2007; Escárcega-Galaz *et al.*, 2018). Chitosan's ionic interaction with alginate has also been applied as adhesives for ostomy bags (Pan, Matsuda and Yuk, 2020). Chitosan interacts with oppositely charged components of the extracellular matrix and growth factors, making it interesting for tissue regeneration and scaffolds (Escárcega-Galaz *et al.*, 2018).

In its application for tissue regeneration scaffolds including bone regeneration, it can interact with host tissues and can incorporate other compounds. For example with bone regeneration, osteo inductive molecules such as strontium and hydroxyapatite are included to facilitate mineral deposition (Rodríguez-Méndez *et al.*, 2018). Chitosan has also been incorporated into guided tissue regeneration techniques where it has been used as a self-hardening paste with hydroxyapatite (Rodríguez-Méndez *et al.*, 2018).

Drug delivery systems (pharmaceutical excipient) are based on chitosan's polyelectrolyte character in solution, as well as its mucoadhesive properties where it can interact with positively charged sialic acid residues in mucin (Illum *et al.*, 2001). It has been reported that chitosan has absorption enhancing properties as it can open tight epithelial junctions allowing molecules to pass by mucosal cells (Artursson *et al.*, 1994). With absorption enhancing properties it has been studied with medicinal compounds that are normally difficult to solubilise and pass into the body (Real *et al.*, 2018). With anionic molecules it can form different polyelectrolyte complexes such as nanoparticles and micelles which allow the encapsulation of molecules. This can be particularly useful for hydrophilic molecules and with the variation in the physicochemical properties, chitosan can interact with negatively charged structures on the surface of cells (Erman and Veranič, 2018), and also with the

glycocalyx which could potentially result in a controlled release mechanism that can be tailored for particular treatments (Erman and Veranič, 2018).

Chitosan's gene delivery applications are similar to drug delivery, free amine groups have a cationic charge which results in the polyelectrolyte complex formation with nucleic acid's phosphate backbone meaning that genes and mRNA can be delivered to certain areas of the body (Santos-Carballal, Fernández and Goycoolea, 2018). This could provide therapies for rare diseases and cancers (Erman and Veranič, 2018; Santos-Carballal, Fernández and Goycoolea, 2018), however the transfection efficiency needs optimising further for mammalian cells.

1.6.2 Wastewater

Chitosan's cationic nature is increasingly of interest for applications in contaminated waste streams, in both aqueous environments as well as soils and sediments. Chitosan has been studied for its ability to chelate with inorganic and organic contaminants and there is also interest in applying it in detection of specific contaminating compounds with sensors. It can chelate metal ions from water and can be used as a flocculant to facilitate precipitation of suspended solid contaminants (Yong *et al.*, 2015). It can also be used to remove other nutrients such as nitrates and phosphates which could have important applications for preventing eutrophication (Palansooriya *et al.*, 2021). Furthermore sensor applications could potentially be developed by immobilising enzymes on chitosan (Yong *et al.*, 2015).

Chitosan has been investigated for use in different industries. In the papermaking industry, it is used in a range of applications. It can be used for treating wastewater (Primex, 2022). Here it can be used for the adsorption of dyes, humic acids, metal ions and xenobiotics. Its antimicrobial properties are also useful here for the removal of bacterial cells (Song *et al.*, 2018). In aquaculture wastewater, it is popular for its use in removing soluble proteins remaining in the water that can be reused for feed (Pennotec, 2020). A company called Pennotec ® has a project developing an application in this area currently. In addition to this, chitosan can be used as a fining agent for drinks such as wines and in the mining industry, it can also be used to reacquire metals lost in waste (Yong *et al.*, 2015).

1.6.3 Cosmetics

Chitosan has active properties which are relevant to cosmetic applications as well, it can function as a carrier for active ingredients for cosmeceuticals. It has applications for hair, skin and oral health.

In oral health, chitosan and chitosan derivatives have antimicrobial properties that have been investigated against causative agents of dental caries such as *Streptococcus mutans* and a reduction of dental plaque has been associated with its application (Sano *et al.*, 2003). Studies have compared the impacts of chitosan as a mouth rinse and toothpaste against other popular brands and well known antimicrobials in dental care such as chlorhexidine gluconate (Decker *et al.*, 2005). Chitosan's broader range antimicrobial activity versus chlorhexidine's targeting of *S. mutans* was found to be more effective and also improved the function of chlorhexidine suggesting synergy (Decker *et al.*, 2005). It has also been shown to have less of an abrasive effect when compared with fluoride (Ozalp and Tulunoglu, 2014).

In haircare, chitosan can interact with keratin to form films over hair fibres to provide a level of protection against damage and provide both strength and softness to the hair. It can also be used to modify the viscosity of a solution to be applied to hair. Chitin and chitosan nanofibers have also been developed for cosmetics including hair care, and hair regenerative properties have been claimed as a hair loss solution for surface deacetylated chitin nanofibers (Azuma *et al.*, 2019; Gelfuso *et al.*, 2011). Chitosan composites containing hyaluronic acid (HA) and collagen have also been found to enhance hair properties (Sionkowska *et al.*, 2017). There are also a large number of applications for derivatives including glycol chitosan which can be used as a foaming agent and emulsifier for shampoos (Aranaz *et al.*, 2018).

In skincare, chitosan can form polyelectrolyte complexes with HA which is useful for emollient applications for skincare as chitosan's cations interact with the negative charges on the skin surface (Aranaz *et al.*, 2018). The characteristics of the chitosan used influence its activity in these applications, with a low molecular weight and a highly deacetylated polymer being desirable (Aranaz *et al.*, 2018). Many chitosan derivatives have been applied in this area too. Chitin and chitosan has also been investigated as carriers for potential UV protection in sun creams, using it alongside other UV absorbing chromophores such as urocanic acid (Gomaa *et al.*, 2010; Ito *et al.*, 2015). Chitosan can also be used for its viscosity, surfactant and emulsion properties.

1.6.4 Agriculture

In agriculture, chitosan has been studied for plant stimulatory effects for decades. Chitosan oligomers induce defence responses to physical as well as pathogenic damage in plants dependent on their DDA and degree of polymerisation (Cabrera *et al.*, 2006). Chitosan can trigger pathways for tolerance to abiotic stress, enhances plant growth and yield (Pichyangkura and Chadchawan, 2015). It can increase the shelf life of fruits and flowers as well as activating production of secondary metabolites (Pichyangkura and Chadchawan, 2015). In addition, chitosan could potentially serve as a carrier of active insecticides to prevent other plant diseases.

Chitosan's antimicrobial properties have been shown to be effective against several plant pathogens through the use of seed coatings and foliar spraying. An example of this is its ability to minimise anthracnose (a fungal disease) in a number of different vegetables (Ali A. *et al.*, 2010; Maqbool *et al.*, 2010) and has impacts on nematode infections (Escudero *et al.*, 2016; Escudero *et al.*, 2017; Mwaheb *et al.*, 2017). This could be useful to reduce crop loss due to disease without the need for pesticides. Hazardous herbicide/pesticide use is a significant issue for plant based crops and is the main hazard highlighted on HorizonScan (2021) for import refusals and safety investigations. HorizonScan is an online platform which enables users to monitor food safety issues. Table 23, 24a-c in Appendix A indicate the top hazards for different commodity groups.

While the mechanism of action is still not fully understood, chitosan's antifungal properties differ depending on the fungal species (Verlee, Mincke and Stevens, 2017; Younes *et al.*, 2014). Chitosan could be beneficial to certain fungal species whilst inhibitory to others. In combination with the antibacterial effects, this may have significant impacts on the microbial communities of soils, which could result in both negative and positive impacts for crop cultivation.

Chitosan induces higher production of crops such as strawberries (Mukta *et al.*, 2017), increases the size of potato tubers (Falcón-Rodríguez *et al.*, 2017), and increases in plant size and photosynthetic rate for many species (Malerba and Cerana, 2018). In addition, when plants were subject to abiotic stresses, it has been shown that chitosan can induce tolerance mechanisms. In sweet basil, plant growth parameters were increased and in white cloves

increases in stress protective metabolites were measured (Li *et al.*, 2017; Pirbalouti *et al.*, 2017).

The antimicrobial, film forming and mild antioxidant properties of chitosan have also been applied to extend the shelf life of highly perishable crops, including fresh vegetables and fruits, through the use of edible films and coatings (Elsabee and Abdou, 2013).

1.6.5 Food and drink

In addition to chitosan's application as a fining agent in drinks, it can be applied further into the food and drinks sector like many other biologically derived polymers. Chitosan also has several food applications including preservation, emulsifier, dietary ingredient and for edible coatings and films for food protection. Chitosan's fat binding properties have been linked to use as a way to reduce fat absorption in the gut (Aranaz *et al.*, 2009; Sugano *et al.*, 1980). However, this is not easy to confirm due to the fact that chitosan is able to interact with many molecules and bacteria in the human gastrointestinal tract.

Further to chitosan's application in agriculture, it has also been shown to enhance the shelf life of fruits and vegetables. This has led to increased research on ways to increase the shelf life of highly perishable foods by managing postharvest disease without pesticide residues. Production of edible coatings and packaging films helps reduce microbial degradation, decreases weight/water loss and therefore maintains marketable quality of food for longer durations (Malerba and Cerana, 2018). However, while the antioxidant activity of chitosan has been discussed (Aranaz *et al.*, 2009), the use of chitosan alone in food contact materials has not yet effectively been shown to reduce the oxidation process leading to deterioration in quality of foods. Therefore, a significant amount of research is now focussing on incorporating other bioactive compounds into chitosan edible coatings and films in order to improve its preservative and protective properties. However, research also needs to be cautious not to reduce chitosan's original activity or add additional risks in terms of food safety.

1.7 Edible films and coatings

Several biodegradable molecules including alginate, poly lactic acid (PLA), polyhydroxyalkanoates, cellulose, soy protein, locust bean gum and chitosan have been investigated for extending the shelf life of foods through edible coatings and films. Edible coatings can be used as a solution and the product for preservation can be dipped into or sprayed with the solution for coating (Wang, Qian and Ding, 2018). Alternatively, films can be produced by solvent casting or nanofiber production through electrospinning (Lago *et al.*, 2014; Liu *et al.*, 2017). However, weak mechanical properties and large investment costs restrict the application and so research and development into composite materials is increasing even more rapidly.

1.7.1 Composites

There is increasing interest in composite biomaterials for packaging. These include materials that are targeting improvement in the mechanical and barrier properties, antimicrobial activities and composites for making active packaging materials with additional properties which the biopolymer itself cannot provide. Research into PLA has been significant. Starch and PLA composites have desirable mechanical and barrier properties when compared to individual biopolymer performance (Muller, González-Martínez and Chiralt, 2017). Ali *et al.* (2016) demonstrated an enhancement of the thermal stability of PLA with epoxidized palm oil, and Aytac *et al.* (2017) incorporated antioxidants into PLA to reduce oxidation of foods protected by this material. However, many studies do not compare the performance of biopolymers with a known standard such as a plastic already used in packaging, therefore, having a reference point on how effective the packaging is can be difficult to establish.

1.7.2 Active and intelligent packaging

A potentially useful application for packaging films is for active packaging, the ability to assess the quality of the packaged food through pH sensitive molecules and detecting microbial presence. Recent studies in pH-sensing packaging have focussed on anthocyanin-based extracts such as soy bean seed coating (Wang *et al.*, 2019). Anthocyanins are phenolic compounds that, besides from the pH sensitive colour changes, exhibit antioxidant, anti-inflammatory and anticarcinogenic properties (Wang *et al.*, 2019). The pH colour changes occur due to structural modifications in the presence of an acidic/alkaline environment

(Wang *et al.*, 2019) which would allow food spoilage (often linked to pH change) to be detected. Detecting microorganisms more specifically by sensors for enzymes produced by the organisms have also been studied. Studies have used sensors to detect *Escherichia coli* through an enzyme which is produced by 98% of strains, β-glucuronidase (Ebrahimi, Voss and Schönherr, 2015). In addition to this, there has been some research into protecting light sensitive foods from photo-oxidation (Ahmed and Ikram, 2016; Vilela *et al.*, 2017). These studies added gelatine (Ahmed and Ikram, 2016) and ellagic acid (Vilela *et al.*, 2017) to chitosan films to provide a reduction in light transmittance.

1.7.3 Limitations

A limitation in the literature for the development of these composite materials is the assessment of biodegradability and safety which is not often tested. However packaging companies like Eco-Craft ® (Eco-Craft, 2019) and Natural Bag ® (NaturalBag, 2019) have gained accreditations, such as EN 13432 for compostable packaging (Standards, 2019), for cellulose and corn starch materials respectively. There is also a safety concern with these research materials that are being produced, as they may include proteins and contaminating compounds that could cause harm to consumers. Therefore, it is essential that accreditation and testing are regularly assessed in tandem with this sector of research to ensure consumer safety before being trialled with humans.

1.7.4 Chitosan edible coatings

Chitosan has been involved in edible coating research for extending the shelf life of certain foods. Chitosan's antimicrobial effects have been evidenced repeatedly in this area. Chitosan edible coatings have been shown to reduce the quantity of harmful and spoilage associated pathogens on many perishable items including different fruits, vegetables, meats and seafood (Elsabee and Abdou, 2013). They have also been applied to processed food items including sliced fruit (Poverenov *et al.*, 2014b). Perishable foods are not only prone to microbiological decay, they are also susceptible to a deterioration in nutritional quality.

Fruits, vegetables, meats and seafood are highly perishable with short shelf lives, meaning they are highly prone to rapid deterioration in safety and quality. Highly perishable items contribute largely to food waste (Figure 4) (Parry, Bleazard and Okawa, 2015). 21% of food

waste is due to spoilage (Parry, Bleazard and Okawa, 2015). Therefore, being able to extend the shelf life of foods which are prone to rapid deterioration will help to minimise food waste.

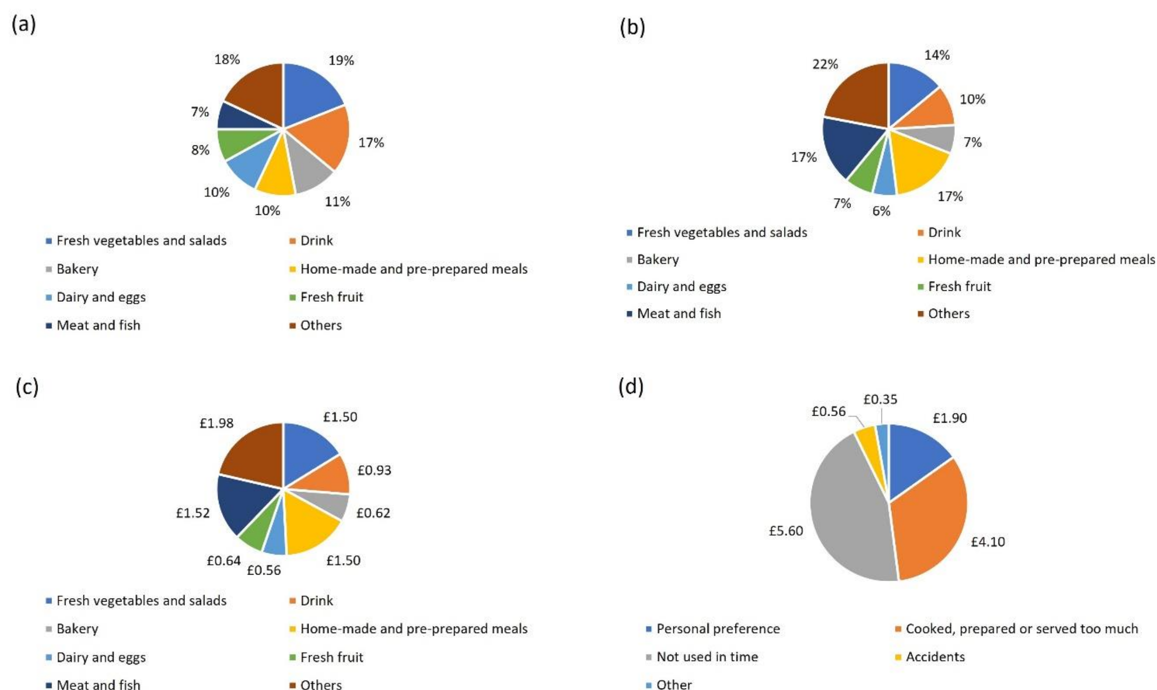

Figure 4- How perishable items contribute to food waste data in the United Kingdom (Parry, Bleazard and Okawa, 2015; WRAP, 2012). (a) Proportions of avoidable food waste by weight (%). (b) Proportions of avoidable food waste by cost (%). (c) Cost of avoidable food waste per household per week (£). (d) Cost of avoidable food waste by reason for disposal (£).

Research has more recently began looking at improving other parts of the preservative process, and to improve the quality of the foods. This includes the contents of nutrients such as ascorbic acid and anthocyanins, the pH, moisture content and antioxidant qualities, as well as sensory features such as texture and taste.

Like films, chitosan has been used for composites with other biopolymers like alginate (Kim, Hong and Oh, 2018), compounds such as carotenoproteins (Hajji *et al.*, 2018; Hamdi *et al.*, 2018), nano materials (Li *et al.*, 2021; Sami *et al.*, 2021) and food waste materials like pomegranate peel (Kharchoufi *et al.*, 2018). Incorporating nutrients into chitosan coatings is also a research area in development.

Differently to antimicrobial impacts, the qualities of the target food are more challenging to measure and they often require methods specific to the food type. In the literature, studies measuring the same qualities often use different biochemical methods which can make it difficult to accurately compare the effects. This highlights the need for established methods for analysing the qualities of coated foods.

1.7.4.1 Weaknesses to chitosan edible coating studies

An essential part often missing from the studies of chitosan in food applications is characterising the properties of the chitosan before using it. Many studies do not measure or state the degree of acetylation or molecular weight of chitosans used. This can lead to variability in reported performance of films and a poor understanding of how the chitosan properties affect the food contact material being studied. As well, it crucially makes the studies impossible to reproduce. For example, molecular weight impacts the viscosity of the coating solution and is going to impact the quantity of coating remaining on the dipped food. Degree of acetylation is widely accepted to have an increased antimicrobial effect when the acetylation is lower (Verlee, Mincke and Stevens, 2017; Younes *et al.*, 2014), and stoichiometrically, different concentrations of acid are going to be required to solubilise chitosan effectively without excess acid remaining present, therefore reporting this is also crucial. Degree of acetylation is going to be increasingly important as the proportion of free amine groups play an important role in chitosan's solubility and polyelectrolyte behaviour (Rinaudo, 2006). This means that when using complementary (anionic) molecules in composites (e.g. alginate and pectin) there may be differences in the way they interact when there is a highly deacetylated chitosan versus a more acetylated chitosan.

In addition, studies often also include poor controls for isolating the impact of the chitosan itself from the chemicals used to induce its dissolution. Further still, the pH of the coatings vary widely. This can lead to an excess amount of acid being used in the coating solution which may distort results.

1.8 Achieving the UN's Sustainable Development Goals

With the world population rising, the need for secure and sustainable food production is present. The need for protein to sustain the population without putting strain on other resources has been researched. Two developing areas in research that could be fundamental to support global progression in achieving several of the UN's Sustainable Development Goals (Figure 5) are insect farming and marine aquaculture.

Figure 5- The 17 UN Sustainable Development Goals

1.8.1 Blue biotechnology

Solutions to some of the issues highlighted that contribute to global warming can be facilitated by developing more sophisticated approaches to blue biotechnology. Aquaculture, on different trophic scales, offers several solutions to key issues surrounding climate change. This is because species are used that have no fresh water requirements, they are capable of water filtration and carbon sequestration, and are not competing for space on land. Filter feeding bivalves offer ways to help reduce waters overloaded with nutrients and seaweeds are capable of sequestering carbon in large quantities (Duarte *et al.*, 2017). These advantages also come with further opportunities to supply products such as fucans, alginate and carrageenans in significant quantities. Furthermore, they can be a source of novel bioactive molecules for applications in foods, pharmaceuticals, cosmetics and potential biofuels (Buschmann and Camus, 2019).

Aquaculture as a whole can build up from lower trophic levels to species which may be consumed by humans as a protein source. These include fish, crabs, lobster and prawns. Some current aquaculture possibilities also intertwine with insect farming. Insects are a natural part of the diet of many species of fish and therefore insect farming has been investigated for supplying nutrition for fish feeds.

1.8.2 Yellow biotechnology

There has been development of insect farming to supply the vast need for protein to include in animal feed. Animal feed from crops uses a considerable portion of the worlds agricultural land to supply feed as well as a sizeable quantity of water. This puts a strain on sustainability, especially as the population continues to grow and the demand becomes larger.

Like with many of the marine species, insect farming offers solutions to the UNs Sustainable Development Goals. Many species can be reared on different feed substrates in a process called insect bioconversion, helping recover nutrients from lost waste streams to re-enter the food chain. Insects have also been used for their medicinal properties in traditional Chinese medicine with records in The Grand Compendium of Materia Medica (Li Shizhen) during the 16th century, Ming dynasty (Chu *et al.*, 2013). More specifically *Musca domestica*, a holometabolous species, is still used clinically today (Chu *et al.*, 2013).

Insects as a source of animal and aquaculture feed protein have been investigated with various species (van Huis, 2013) and the chemical safety assessed for several dipterans (Charlton *et al.*, 2015). Insects offer advantages over other sources. They have short life cycles which means it is easy to generate a large biomass in a short time frame (van Huis, 2013). They have low water requirements and generally do not need to be reared on large areas of land (van Huis, 2013). They also have low greenhouse gas emissions and good feed conversion ratios (van Huis, 2013). In some countries, insects are already consumed by humans as they are a great nutritional source, however westernised cultures may not be ready to take on direct insect consumption in the immediate future due to their association with dirt and disease (Harvey, 2019). This means there could be more interest and public acceptance in breaking down the insects into different molecules (Caligiani *et al.*, 2018). These positives, as well as the fact that some insects can feed on organic waste (van Huis, 2013), mean that they could be ideal for a circular bioeconomy (EC, 2015a; EC, 2015b).

1.8.3 Arthropods are a source of chitin

Arthropods are an extremely diverse phylum including terrestrial, aquatic and volant animals. They are efficient, adaptable and endure some of the most extreme environments on Earth. More relevantly both insect farming and aquaculture are capable of producing arthropods on large scales in a sustainable manner, while helping achieve the UNs Sustainable Development Goals. Chitin, is a biopolymer derived most abundantly from arthropod species. Although in nature the abundance of fungal chitin may rival this, the chitin-glucan complex associated with fungal chitin may make chitin isolation more challenging. Currently the most commercially produced source of chitin is from marine shellfish such as prawn waste, where the chitin rich exoskeleton is often removed during processing, offering value for both food production and chitin production. Therefore, finding ways of applying chitin and its derivatives are of great interest. In addition, novel methods to extract and purify chitin from glucan in fungal cell walls are being explored (Alimi *et al.*, 2023; Hassainia, Satha and Boufi, 2018; Ivshina *et al.*, 2009).

1.9 Insect chitin biosynthesis

1.9.1 Chitin sources

Chitin is present in insects, crustaceans, as well as other invertebrates such as molluscs, nematodes, and sponges (Merzendorfer, 2011). It is also present in microbes such as fungi, protists, algae and some bacteria (Merzendorfer, 2011). The most abundant sources of chitin are fungi, crustaceans and insects. These taxa are some of the most species rich taxa too. Crustaceans are the most common source of commercially available chitin due to the chitin rich exoskeleton being a significant by-product of human seafood consumption.

Unlike shellfish derived chitin, insects and fungi are not as dependent on seasonality for high production. Insects offer an interesting source of chitin due to the moulting of exoskeletons during metamorphosis (holometabolous insects), offering a source of chitinous material with no other known uses. The different life stages offer opportunities to optimise the production of insects to prioritise certain molecules such as fats, proteins or chitin (Caligiani *et al.*, 2018; Caligiani *et al.*, 2019; Leni, Caligiani and Sforza, 2019). For example chrysalis and pupal exuviae have had chitin extracted from them (Hahn *et al.*, 2020; Luo *et al.*, 2019).

1.9.2 Chitin purpose

In nature, chitin is a heteropolymer of *N*-acetyl-ᴅ-glucosamine and ᴅ-glucosamine. Chitin is structurally similar to cellulose apart from the acetyl and amino group. A number of enzymes show activities for both chitin and cellulose (Hoell, Vaaje-Kolstad and Eijsink, 2010). It is abundant in the exoskeleton of insects where it is subject to dynamic changes and turnover which allow its structure and properties to be adapted with other compounds such as lipids, minerals, catecholamines and proteins, to provide optimal functional characteristics for certain parts of anatomy (Andersen, 2010; Merzendorfer, 2011; Merzendorfer and Zimoch, 2003; Moussian, 2010; Zhu *et al.*, 2016). These processes and modifications are all controlled by an array of specialised enzymes.

Chitin polymers are formed in three main steps from glucose, glycogen or trehalose as a starting substrate (Muzzarelli, 2011). This is enzymatically transformed into *N*-acetyl-β-ᴅ-glucosamine (GlcNAc) residues as depicted in Figure 6. Then GlcNAc is modified to form active uridine diphosphate (UDP)-GlcNAc residues with the UDP-GlcNAc pyrophosphorylase enzyme (Muzzarelli, 2011; Zhu *et al.*, 2016). With the presence of a divalent cation, the UDP

GlcNAc is polymerised by chitin synthase enzymes, which are then exported to the extracellular space where they aggregate in an antiparallel arrangement to form alpha chitin microfibrils (Muzzarelli, 2011; Zhu *et al.*, 2016). Alpha chitin microfibrils provide strength to the exoskeleton due to extensive hydrogen bonding between individual polymer chains (Zhu *et al.*, 2016).

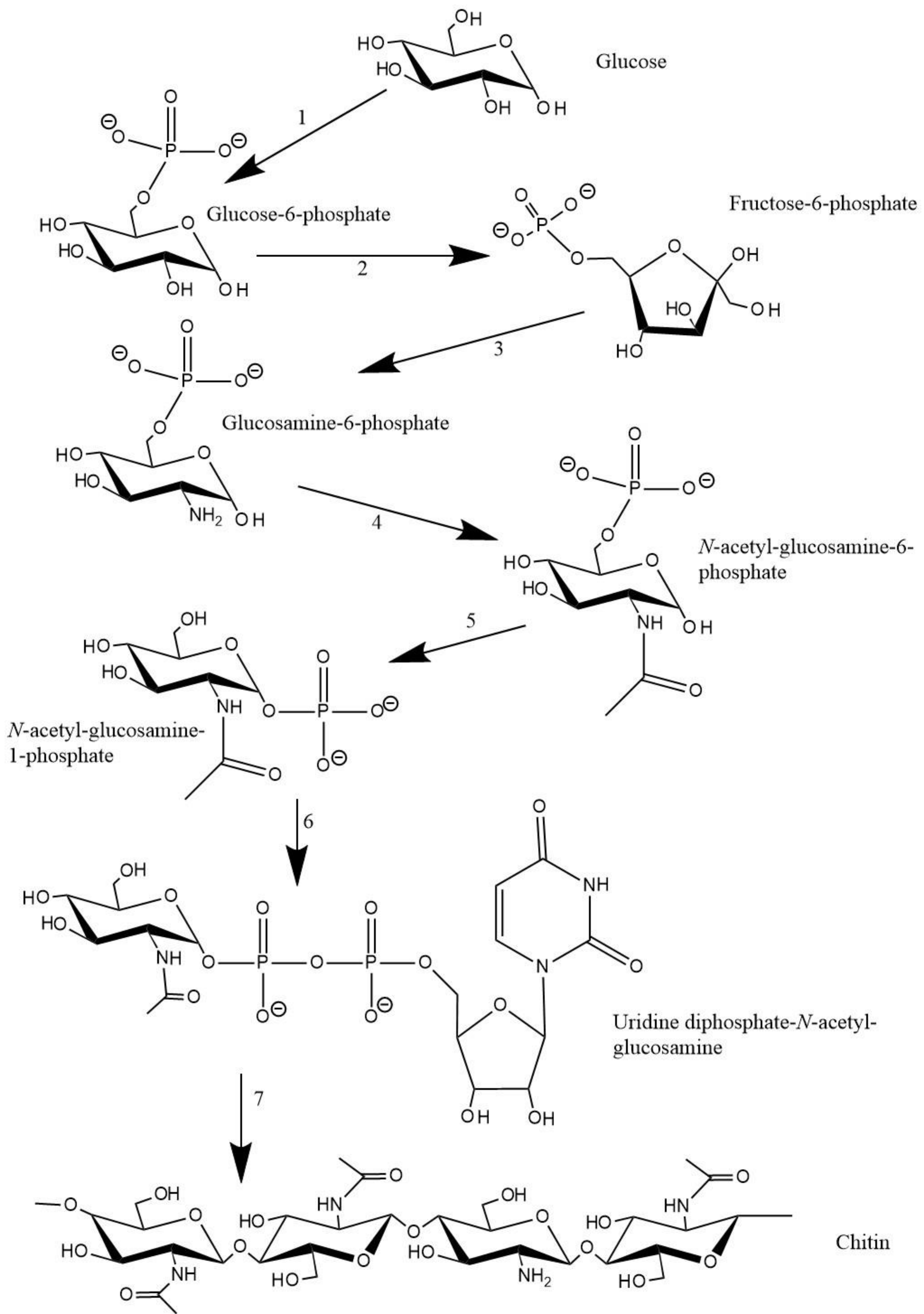
Glucose
1
Glucose-6-phosphate
2
Fructose-6-phosphate
3
Glucosamine-6-phosphate
4
N-acetyl-glucosamine-6-phosphate
5
N-acetyl-glucosamine-1-phosphate
6
Uridine diphosphate-N-acetyl-glucosamine
7
Chitin

Figure 6 – The pathway for enzymatic formation of chitin from glucose (Cohen, 2001; Kramer and Koga, 1986; Merzendorfer and Zimoch, 2003). Glucose is transformed into glucose-6-phosphate by hexokinase (1). Glucose-6-phosphate is transformed into fructose-6-phosphate by glucose-6-phosphate isomerase (2). Fructose-6-phosphate is transformed into glucosamine-6-phosphate by glutamine:fructose-6-phosphate aminotransferase (3). Glucosamine-6-phosphate becomes *N*-acetyl-glucosamine-6-phosphate through the action of glucosamine-6-phosphate *N*-acetyltransferase (4). *N*-acetyl-glucosamine-6-phosphate is transformed into *N*-acetyl-glucosamine-1-phosphate by phosphoacetylglucosamine mutase (5). *N*-acetyl-glucosamine-1-phosphate becomes Uridine diphosphate-*N*-acetyl-glucosamine (UDP-GlcNAc) through the action of UDP-GlcNAc pyrophosphorylase (6). The active UDP-GlcNAc is polymerised into chitin through the action of chitin synthase (7).

In holometabolous insects, which progress through complete metamorphosis, further development steps are taken to adapt the cuticle. Sclerotization, quinone tanning and melanisation can begin in the prepupae of some dipterans or otherwise in the imago, the adult/final life stage (Andersen, 2010; Merzendorfer, 2011; Moussian, 2010; Zhu *et al.*, 2016). This involves the crosslinking of proteins and chitin microfibrils with *O*-quinones derived from tyrosine, providing further stabilisation of the cuticle (Andersen, 2010; Merzendorfer, 2011; Moussian, 2010; Zhu *et al.*, 2016).

1.9.3 Chitin degradation in nature

Due to the abundance of chitin in nature, numerous taxa have genes relevant to chitin degradation. *Serratia marcescens*, a species of gram negative bacteria has received a lot of research attention leading to significant detail of its chitin degradation process (Vaaje-Kolstad *et al.*, 2013). In brief, a series of chitinases with different actions disintegrate chitin into its monomers. The chitinases have limited functionality due to the crystalline nature of chitin fibrils (Vaaje-Kolstad *et al.*, 2013). In order to make chitin more readily available, enzymes called lytic polysaccharide monooxygenases (LPMOs) break up glycosidic bonds in chitin chains to reduce the crystallinity and provide more reducing and non-reducing ends for the chitinases to act upon (Vaaje-Kolstad *et al.*, 2013). LPMOs are classified as auxillary activity enzymes in the CAZy database and are part of families AA9-11, AA13-15.

1.9.4 Chitin deacetylation in nature

Chitin is a heteropolymer in nature and therefore consists of a proportion of deacetylated residues. Chitin deacetylases (CDAs) are needed to remove the acetyl groups and these are present in most chitin containing organisms. They have different functions depending on taxa. Chitin deacetylases are part of the carbohydrate esterase family 4 (Hoell, Vaaje-Kolstad and Eijsink, 2010).

In fungi, chitosan is believed to have a stealth role in fungi-plant interactions for pathogenicity and symbionts. Chitin produces an immune response in plants and by altering the main binding site (*N*-acetyl group) for the plants detection of chitin, fungi are able to survive within the plant (Gao *et al.*, 2019).

In insects, chitin deacetylases have another crucial role for survival and are categorised into five structural groups. They are believed to have different functional roles. For example gene knockdowns for some groups of CDAs have been shown to be essential to survival in insects and cause significant disorders in moulting which ultimately leads to death (Zhu *et al.*, 2016). Some have a more localised role in the insect similarly to insect chitin synthase groups (Zhu *et al.*, 2016).

1.9.5 Chitosan natural purpose

Chitin and chitosan oligomers are involved in cell signalling and can elicit immune responses in plants (Cabrera *et al.*, 2006). Rhizobial bacteria secret fatty acid linked chitin oligomers for nodulation signalling, triggering symbiotic responses in leguminous hosts (Merzendorfer, 2011; Peters, 1997).

Chitosan plays a role in fungal infections which facilitates the pathogenicity of spores (Escudero *et al.*, 2016). Chitosan can also disrupt calcium complexes formed with homogalacturonan in pectin during plant infections as well oligogalacturonides which the plant produces as defence (Cabrera *et al.*, 2010).

1.10 Insect chitin extracted: Common properties and application studies

1.10.1 Isolating chitin

Chitin has been extracted from a number of different taxa. Typically this consists of sequential treatment with an acid (to remove minerals) and then a base (to remove proteins) or vice versa. Two main arrangements of chains within microfibrils have been studied: Alpha and beta chitin. Alpha is the most abundant in nature and the most comprehensively hydrogen bonded making it highly crystalline (Roberts, 1992).

It has been suggested that the properties of chitin depend on its source (Hajji *et al.*, 2014). While this is true in the sense that different isoforms can come from different sources, it is much more likely that the differences being measured are actually differences in the purity of the chitin. This is due to the fact that different chitinous sources will have different compositions of chitin and other molecules in the material, depending on the life stage, diet and conditions the organism has been grown in. The extraction procedure between different chitinous material sources has not been optimised for specific substrates. There is also a need for quantification of yields of chitin and chitosan and further clarity into the purity of the extracted material (Hahn *et al.*, 2020).

The conventional extraction of chitin is known for using a significant proportion of water in the extractions, therefore a lot of recent research has tried to optimise this process using strategies such as enzymatic processing, ionic liquids and deep eutectic solvents (Qin *et al.*, 2010; Sharma *et al.*, 2013; Younes and Rinaudo, 2015; Zhou *et al.*, 2019). However, considering chitin's use in applications, it is important to note that the purity of chitin is extremely important due to the fact that some chitin containing organisms possess tropomyosin proteins which provoke a severe immune response in some humans (Muzzarelli, 2010). While the intensive chemical processing used to acquire chitosan is likely to eradicate any residual proteins present in the extracted chitinous material, there is a need for confirmation of the purity of the chitosan before it can be safely applied further.

1.10.2 Isolating chitin from insects

The use of insects for animal feed purposes has gained increasing attention as alternative sources of protein are sought together with sustainable production methods. The focus at both research and commercial production levels has primarily been on larvae and prepupae

of the black soldier fly (BSF), *Hermetia illucens* and larvae of the yellow mealworm *Tenebrio molitor*. Larvae of the house fly, *Musca domestica*, and the lesser mealworm, *Alphitobius diaperinus*, have also been researched.

1.10.3 Isolating chitin from specific taxa

One species of insect that can be fed on waste is the BSF (*Hermetia illucens*). This is a saprophagous, holometabolous species of the Dipteran order (Bulak *et al.*, 2018). The larvae grow on a range of substrates including food waste and human faeces (Banks, Gibson and Cameron, 2014). Other advantages are that the prepupae are self-harvesting and the adult life stage relies entirely on fat stored during the larval phase and therefore does not feed, limiting it as a vector for disease. There has been interest in BSF in the literature for food applications (Wang and Shelomi, 2017), entoremediation (Bulak *et al.*, 2018) and using BSF proteins as a bioplastic (Barbi *et al.*, 2018). This has led to a number of commercial companies that produce them. Some of these companies include SFly ®, Symton ®, and Enterra ®. The insects can be sold whole, but for better value there is interest in developing fractionation and uses for the different molecules that are present. Caligiani *et al.* (2018) extracted fractions of lipids, proteins and a chitin fraction from BSF. The chemical methods proved most effective for salvaging distinct fractions, but the use of an enzyme-based extraction showed promise and the authors concluded that it was worthy of further investigation. The fractions containing different molecules can then be used for different applications including the proteins for animal feed, fats potentially for biodiesel and chitin for various applications, including when converted to chitosan. But the variability with all stages of the process is not well studied and the way procedures affect yields of each fraction is unclear. Studies (Caligiani *et al.*, 2019; Leni, Caligiani and Sforza, 2019) have looked into the effects of killing method and found differences in the protein and lipid fractions. This highlights the variability and more studies are needed to fully understand the implications of the different stages of the process on the products.

The least studied fraction of molecules from BSF is chitin. This may be due to the yield from the flies which is relatively low (Caligiani *et al.*, 2018). However, unlike more popular sources of chitin, the flies can be grown in controlled conditions and it could be possible to accurately assess how the whole process and its variables are affecting the overall product properties.

For chitin extraction from BSF, a similar chemical process to shellfish has been used (Figure 7).

Figure 7 – Basic chemical processing of raw chitin rich material to produce chitin.

The chitin has been examined by FTIR, X-ray diffraction and microscopy (Waśko *et al.*, 2016). Furthermore chitosan has been produced from BSF (Hahn *et al.*, 2020).

1.11 Focus

1.11.1 Yellow and blue biotechnology.

Black soldier fly are researched for converting food waste into protein that can help in supplying feed to animals more commonly consumed. Black soldier fly life stages have recalcitrant material rich in chitin which, when derived from other sources, is proving to be a valuable biopolymer due to wide ranging applications. However, compared to conventional sources of chitin such as shellfish waste, chitin rich insect waste is harder to separate in a good concentration. Prepupae are self-harvesting which makes them a viable source for fractionation of molecules, chitosans from the chitinous fraction have not been characterised.

1.11.2 Insect bioconversion

Insect bioconversion could reduce food waste on two levels. By converting wasted food into useful protein, but also by contributing to the supply of more chitin-rich materials that could be used to produce chitosans and increase the shelf life of highly perishable, high value protein for human consumption. This could be by supplying more insect derived chitinous material directly or through applying the insect protein as a feed to other chitinous organisms such as shellfish aquaculture.

1.11.3 Understanding a chitosan's antimicrobial action

Chitosans have evident antimicrobial activity but the mode of action remains speculated which limits our understanding when applying the molecules because assumptions are made that often are not exact and result in variability that cannot be properly explained. If mode of action varies for different chitosan characteristics, it is fundamental that methods are implemented that can adequately characterise the polymer used in a study. Having methods easily available on limited budgets is also important, as it widens the opportunity for labs with lower budgets to participate in the research field and diversify ideas.

1.11.4 Chitosan and shelf life extension

Chitosans alone have been studied for reducing spoilage in combination with some other treatments such as modified atmosphere packaging and additive compounds that introduce

greater qualities to the coatings (Latou *et al.*, 2014). Modified atmosphere packaging uses plastics to contain the atmosphere and so relying on it as a method to extend shelf life contradicts the aim of reducing plastics. Additive compounds used in studies may have pungent sensory characteristics that are not pleasant for human consumption.

Furthermore, there is a need for more detailed justification for applying the coating to certain food types and properly identifying the chitosans functional role. For example the antimicrobial activity of chitosan and which organisms are more susceptible. Despite the speculative literature suggesting chitosans characteristic dependent antimicrobial action, there is no justification provided for using a chitosan with 'X' characteristics over 'Y'.

1.11.5 Aims and objectives

The aims of this work were to extract chitosan from the black soldier fly (*Hermetia illucens*) to understand the quantity of chitosan available from a given life stage. It was also important to identify the characteristics of the chitosan produced from the black soldier fly so that the impacts the characteristics may have on later experiments could be understood. This includes the average degree of acetylation of the chitosan produced as well as the molecular weight distribution. A need for a cheap, easy, comparative method for molecular weight analysis in the lab was identified.

Furthermore to understand the suitability for chitosan as an antimicrobial coating, chitosans were subject to antimicrobial susceptibility tests to identify bacteria which chitosan inhibits and bacteria that are not inhibited. Additionally chitosans with different characteristics were screened against a panel of *Bacillus* reporter strains to provide an opportunity to evidence whether there are differences in the way *Bacillus* reacts to chitosan exposure.

The outcome of the antimicrobial susceptibility tests help direct the chitosan application to a target food group, along with an understanding of the risks associated with different food groups. Using chitosans with different characteristics on foods to evidence the difference the characteristics may have is also a neglected gap in the literature. Therefore using considerably different chitosans could be useful to highlight any beneficial characteristics.

The **aims** and objectives for the thesis are further summarised:

1 Extract chitin from the black soldier fly

1.1 Extract chitin comparing two chemical based methods on a small scale

1.2 Increase the scale of the extraction to provide chitosan on a gram scale.

2 Deacetylate chitin and characterise the chitosan

2.1 Degree of acetylation characterisation

2.2 Develop an affordable method for measuring molecular weight of chitosans in the lab

2.2.1 Calibration of the specific system developed

2.3 Characterise the molecular weight of chitosans

3 Assess the antimicrobial activity of chitosan

3.1 Develop antimicrobial susceptibility tests to *Escherichia coli* and S*taphylococcus aureus*

3.2 Measure the antimicrobial activity of chitosan to *Bacillus subtilis*

3.3 Measure the antimicrobial of chitosan against a selection of foodborne bacteria

3.4 Compare the responses of a panel of *Bacillus* reporter strains to different chitosans

4 Apply chitosan as a coating material to a food to reduce spoilage

4.1 Collect information regarding the perishable nature of foods using HorizonScan (2021)

4.2 Construct a narrative towards a specific food group utilising information gathered from HorizonScan (2021) and antimicrobial susceptibility tests

4.3 Measure the effects of different coatings on a food using total viable counts (TVC), thiobarbituric acid reactive substances (TBARS) and pH

4.4 Measure the effects of a chitosan compared to the control to determine if there are significant differences in TVC TBARS and pH values.

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Chapter 2. Chitosan production and characterisation

2.1 Introduction

2.1.1 Chitin extraction

Extracting chitin from biomass has conventionally involved treatment with a base to remove proteins and an acid to remove minerals. Previous studies on chitin extractions from insects are based on chitin extractions from other sources. There are a variety of chemical concentrations and conditions that are used for specific steps of the extraction and it appears that the different steps can be performed in different orders. The most common steps applied are defatting, deproteinisation, demineralisation and decolouration.

Insect chitin extraction procedures have been the subject of research for over 10 years now. A summary of some reported extraction methods and yields is provided in Table 1. Nemtsev *et al.* (2004) and Draczynski (2008) extracted chitin from honeybees (*Apis mellifera*). Nemtsev *et al.* (2004) used NaOH at varying concentrations followed by a decolouration (H_2O_2) step to extract chitin achieving yields between 19 and 37%, while Draczynski (2008) used successive acid (1 M HCl), alkali (1 M NaOH) and decolouration ($KMnO_4$/oxalic acid) steps achieving 18.3% chitin yield. Ai *et al.* (2008) and Jing *et al.* (2007) extracted chitin from housefly larvae (*Musca domestica*). Similarly to Nemtsev *et al.* (2004), Ai *et al.* (2008) did not use an acid treatment to demineralise. Ai *et al.* (2008) used 1 M NaOH then a colouration ($KMnO_4$/oxalic acid) step. Jing *et al.* (2007) used 5% NaOH then decolouration ($KMnO_4$/oxalic acid) followed by an acid (1 M HCl) demineralisation step. Neither reported chitin yield in their studies. Liu *et al.* (2012) extracted chitin from *Holotrichia parallela* using deprotein (1 M NaOH), demineral (1 M HCl) and decolourisation ($KMnO_4$/oxalic acid) steps. They achieved a 15% chitin yield. and compared this against several chitin sources. Caligiani *et al.* (2018) extracted chitin from black soldier fly (*Hermetia illucens*) with a defatting step using petroleum ether followed by 1 M NaOH and 2 M HCl, without decolouration, measuring 11.7-14.6% yields.

Table 1-- A comparison of different chemical chitin extraction methods from different insect species indicating the sequence of chemical treatments and the yield of chitin. Abbreviations are defined as follows: $KMnO_4$- potassium permanganate, CTAB – cetrimonium bromide, ADF/ADL – acid detergent fibre and acid detergent lignin, H_2SO_4 – sulphuric acid, HCl – hydrochloric acid, NaOH – sodium hydroxide.

Reference	Species	Method sequence			Yield (%)
Nemtsev *et al.* 2004	*Apis mellifera*	0.25-19% NaOH		H_2O_2	19 to 37
Draczynski 2008	*Apis mellifera*	1 M HCl	1 M NaOH	$KMnO_4$/oxalic acid	18.3
Ai *et al.* 2008	*Musca domestica*	1 M NaOH		$KMnO_4$/oxalic acid	Not reported
Jing *et al.* 2007	*Musca domestica*	5% NaOH	$KMnO_4$/oxalic acid	1 M HCl	Not reported
Caligiani *et al.* 2018	*Hermetia illucens* (BSF) prepupae	Defat	1 M NaOH	2 M HCl	11.7 to 14.6
Liu *et al.* 2012	*Holotrichia parallela*	1 M NaOH	1 M HCl	$KMnO_4$/oxalic acid	15
Spranghers *et al.* 2017	BSF prepupae	1 M NaOH	1 M HCl	$KMnO_4$/oxalic acid	5.5 to 7
Purkayastha and Sarkar 2020	BSF exuviae and imago	1 M NaOH	1 M HCl	$KMnO_4$/oxalic acid	9
Smets *et al.* 2020	All BSF life stages	Defat	1 M HCl	1 M NaOH	3 to 6
Hahn *et al.* 2018	BSF larva	Defat	0.5 M H_2SO_4 + 20 g L^{-1} CTAB	ADF/ADL	5 to 15
Zhou *et al.* 2019	BSF prepupae skimmed	1 M HCl	2.5 M NaOH		6.5

From this it is clear that there are differences in the interpretation of what is necessary for chitin extraction from insects. Some skip certain steps such as demineralisation and there are differences in the order of the different steps too. Further adaptations of methods need care when optimising to ensure they are not incomplete or otherwise provide overestimates for chitin content.

Accurately measuring chitin content is still problematic. This is due to the presence of nitrogen, which disrupts protein quantification through elemental analysis and provides doubt that chitin purity has been achieved. Gravimetric measurements following the extraction steps still serve as the most common and reliable option in the literature. More recently, Spranghers *et al.* (2017) measured a chitin content ranging between 5.5 and 7% for BSF prepupae using the method from Liu *et al.* (2012). Purkayastha and Sarkar (2020) used the method from Liu *et al.* (2012) and achieved a yield of 9% for BSF exuviae and 23% for imagoes. Caligiani *et al.* (2018) measured a chitin content of 9% for BSF prepupae using a gas chromatography mass spectrometry (GC-MS) hydrolysis based method for quantification of glucosamine residues (Flannery *et al.*, 2001). Smets *et al.* (2020) adapted a biorefinery method from Caligiani *et al.* (2018), which resulted in a 3-6% chitin yield across larval, prepupal and pupal life stages of BSF.

Differently to these, Hahn *et al.* (2018) utilised the acid detergent fibre (ADF) and acid detergent lignin (ADL) methods for plant material to compare with their own method accounting for fibre, acetate and catechols. This measured a yield of 5-15% chitin in BSF larvae. However, there were limited replicates and the precise values were not published. The comparison of three methods made by Hahn *et al.* (2018) could be useful, if the patterns remain when the replicate numbers are increased. This may provide significant value in tailoring chitin extraction procedures for specific substrates.

Each of these methods require several wash phases and subjects the chitin to harsh conditions that could lead to deacetylation and chain degradation (King *et al.*, 2017). Therefore, it is desirable to extract chitin more efficiently, producing less harmful waste, accurate assessments of yields, as well as ensuring a higher quality and quantity of chitin is extracted. There have been two main research routes for more sustainable chitin extractions. These include the use of enzymes and the use of green chemistry methods such as ionic liquids.

Using enzymes to purify chitin is proving to be a challenging procedure which requires several different enzymes and optimal conditions for their activity (Caligiani *et al.*, 2018; Hamdi *et al.*, 2018; Younes *et al.*, 2012; Zhang *et al.*, 2017). An enzymatic method would be extremely useful against the conventional method because it would lead to less chemical waste and avoids the harsh chemical conditions (Zhang *et al.*, 2017). On the other hand, enzymatic extraction also leads to long fermentation times, high costs and still requires optimisation to improve the chitin purity (Zhu *et al.*, 2017).

A great deal of attention in recent years has been dedicated to ionic liquids (IL) for extracting molecules from biomass (Aspras, Jaworska and Górak, 2017; Qin *et al.*, 2010; Shamshina and Berton, 2020). ILs are solids that have a melting point below 100 °C (Qin *et al.*, 2010). Cellulose and chitin have reportedly been dissolved in ILs (Aspras, Jaworska and Górak, 2017; Qin *et al.*, 2010; Shamshina and Berton, 2020) and several papers report chitin extraction from biomass (King *et al.*, 2017; Qin *et al.*, 2010). However, the costs for the reagents to produce ILs are expensive and often have high toxicity (Sharma *et al.*, 2013).

A solution to the high costs and toxicity of ionic liquids is the use of deep eutectic solvents (DES), which operate similarly to ILs. They are composed of two or three chemicals that are capable of association through a hydrogen bond donor and acceptor (Sharma *et al.*, 2013). They have a melting point below 100 °C when combined (Sharma *et al.*, 2013). DES have

reportedly been used in studies for chitin extraction from a range of arthropods including lobster shells, shrimp shells and black soldier flies, but the method details are not well documented (Bradić, Novak and Likozar, 2020; Zhao *et al.*, 2019; Zhou *et al.*, 2019; Zhu *et al.*, 2017). The reagents are significantly cheaper, safer and have no reported alteration to chitin during dissolution (Sharma *et al.*, 2013) but there are considerable differences in the degree of purity achieved.

Previous studies have directly analysed the purity of chitin extracted from biomass through several methods: Black and Schwartz (1950), X-ray diffraction and solid state ^{13}C NMR (ssCNMR) (Bradić et al., 2020; King et al., 2017; Waśko et al., 2016; Zhou et al., 2019). The Black and Schwartz (1950) method involves a similar treatment to the traditional extraction in which the biomass is treated with acid and then alkali. Following this, the material is combusted at high temperature to determine chitin content from loss of mass. A parallel analysis using the Kjeldahl method for nitrogen determination measures the extent to which proteins have been removed. The ssCNMR method for quantifying protein contaminants was developed by King *et al.* (2017). They used bovine serum albumen in a ratio with chitin to produce a calibration curve and then performed various extractions to see how the protein contamination was affected. These two methods were compared and found to produce similar results (King *et al.*, 2017). Furthermore, X-ray diffraction presents the ability to assess whether mineral contaminants remain with the chitin (King *et al.*, 2017; Zhou *et al.*, 2019).

Producing large consistent quantities of pure chitin are necessary for further characterisation by other methods. Therefore Aim 1, Objective 1 was to extract chitin comparing two chemical based methods on a small scale with Aim 1, Objective 2 following on from this to increase the scale of production of the extraction to provide chitosan on a gram scale which would provide enough material for further experimentation.

2.1.2 Degree of acetylation determination

For determining the degree of acetylation (DA) or its inverse the degree of deacetylation (DDA) of chitosans, there are several techniques that are popular in the literature. These include Fourier-transform infrared spectroscopy (FTIR), ^{1}H NMR, ^{13}C NMR, UV spectroscopy, titration and hydrolysis combined with chromatography.

FTIR and titration are two of the most commonly used methods in insect chitosan studies. FTIR is the most common method for DA determination. Samples can be analysed in the solid state. This allows the full range of acetylation values to be measured. Solid state ^{13}C NMR is also useful in this respect but is not so commonly used. FTIR can also be used to differentiate between the different chitin isoforms. However, the sample preparation using potassium bromide (KBr) pellets can have an impact on the band intensities, and with chitosan's hygroscopic nature, it can cause difficulties in achieving accurate results. Previous literature has highlighted hygroscopicity as an important issue with FTIR DA determination due to its influence on certain FTIR bands used in calculating the DA (Baxter *et al.*, 1992; Lavertu *et al.*, 2003; Sabnis and Block, 1997). In addition, FTIR often utilises another method for calibrating the instrument with a chitosan of known DA, which means it is reliant on another DA determination method.

Titration is another commonly used method for DA determination in the literature and particularly so for insect chitosan (Ai *et al.*, 2008; Jing *et al.*, 2007; Hahn *et al.*, 2020; Nemtsev *et al.*, 2004). This is useful as it does not require sophisticated equipment. pH meters are common laboratory equipment compared with NMR and FTIR machinery, titration also requires a calibration curve to be generated (Lavertu *et al.*, 2003). These two methods, most commonly used for determining the DA of insect derived chitosan are therefore dependent on individual preparation, conditions and instrumentation (Lavertu *et al.*, 2003).

^{1}H NMR has been used for DA determination and characterising other binary polysaccharides including alginate composition and pectic HG esterification (Grasdalen, 1983; Vårum *et al.*, 1991a). The main limitation of this method is that samples must be soluble as this only covers a certain range of DA values for chitosans. This can be difficult for chitosans with higher degrees of acetylation, therefore solid state techniques such as FTIR and ^{13}C NMR are also useful for chitosans and chitins with poor solubility and high degrees of acetylation.

^{1}H NMR is an accurate way of calculating DA of fully soluble chitosans. The method also uses a small amount of chitosan (~6 mg) which makes it a useful option. Vårum *et al.* (1991a) used ^{1}H NMR to assess the distribution of acetyl groups remaining on chitosans after homogenous and heterogenous deacetylation reactions and found no differences in distribution patterns. Hirai, Odani and Nakajima (1991) compared ^{1}H NMR with elemental analysis and colloidal titration for a number of chitosan samples and proposed some differences to the experimental parameters. Peaks corresponding to chemical structures have previously been identified (Hirai, Odani and Nakajima, 1991; Vårum *et al.*, 1991a), (Figure 8).

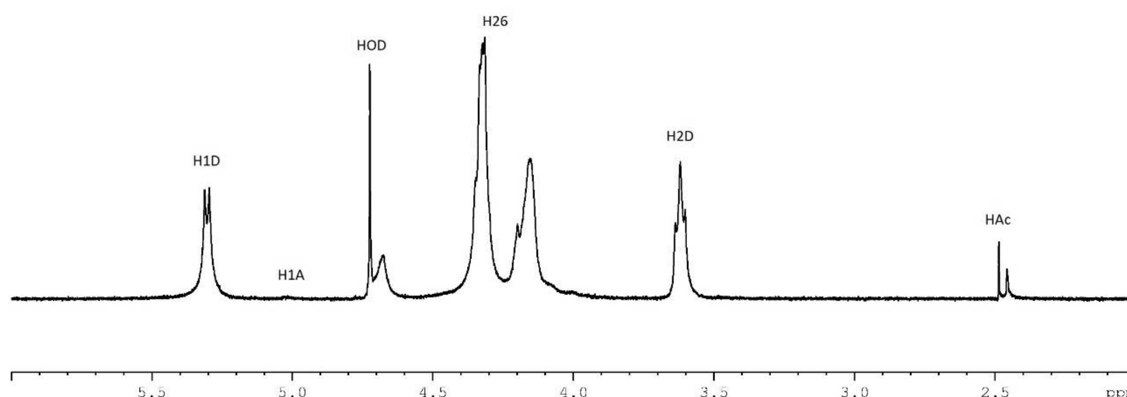


Figure 8 – ^{1}H NMR spectra for black soldier fly derived chitosan highlighting peaks used for DA calculation. H1D = integral of the peak (ppm ~5.3) corresponding to the proton attach to the carbon atom labelled in the Figure 9. HAc = integral of the peak (ppm ~2.45) corresponding to the acetyl protons. H1A = integral of the peak (ppm ~4.9) corresponding to the proton attached to the carbon atom labelled in the Figure 9. H26 = integral of the peaks (~3.8-4.2 ppm) corresponding to the monomer backbone protons.

Lavertu *et al.* (2003) assessed the ^{1}H NMR method for DA determination for different equations (Hirai, Odani and Nakajima, 1991; Vårum *et al.*, 1991a) using different peaks and found that certain equations are better than others for differently acetylated chitosans due to how distinguishable the different peaks are. The equations used were:

1: DDA (%) = (H1D/ (H1D + (HAc/3))) *100

2: DDA (%) = (1-([1/3HAc]/[1/6H26]))*100

3: DDA (%) = (H1D/ (H1D +H1A)) *100

H1D refers to the integral of the peak (ppm ~5.3) corresponding to the proton attach to the carbon atom labelled in Figure 9. HAc refers to the integral of the peak (ppm ~2.45) corresponding to the acetyl protons. H1A refers to the integral of the peak (ppm ~4.9) corresponding to the proton attached to the carbon atom labelled in Figure 9. H26 refers to the integral of the peaks (~3.8-4.2 ppm) corresponding to the monomer backbone protons.

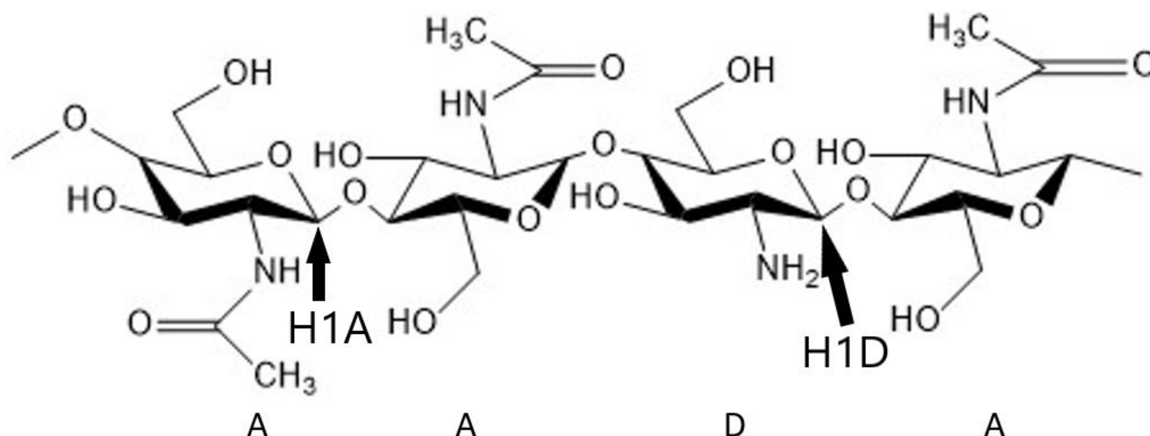


Figure 9 – Chemical structure of four chitin monomers. A – acetylated, D – deacetylated. H1A indicates the position of a hydrogen atom on an *N*-acetyl-D-glucosamine monomer which generates a peak at ~2.45 ppm on a ^{1}H NMR spectra used for calculating DA. H1D indicates the same position but on a D-glucosamine monomer.

Equation 3 is not recommended for chitosans with a high degree of deacetylation (Lavertu *et al.*, 2003). Lavertu *et al.* (2003) concluded that ^{1}H NMR is a suitable method for fast, precise and reproducible DA determination of chitosans and the different equations used have internal consistency.

2.1.3 Deacetylation reactions

Chitosan deacetylation can be performed through different routes of chemical treatment. There are options for both heterogeneous and homogeneous deacetylation conditions.

In heterogeneous deacetylation, chitosan is produced by heating to 100-140 ^{o}C in a strong alkali solution typically consisting of 40-60% (w/v) sodium hydroxide for several hours (Roberts, 1992). Homogenous deacetylation, originally referred to by Kurita *et al.* (1989),

involves long periods of time in strong sodium hydroxide below boiling temperature. Another homogenous approach involving freeze thaw cycles has also been suggested by Nemtsev *et al.* (2002) and more recently used by Hahn *et al.* (2020). This allows the chitin to swell and a solution that has a yellow colour forms. Swelling is believed to reduce the aggregation of the chains to allow more even deacetylation of the chitin (Nemtsev *et al.*, 2002). However, this process is extremely slow and can take several weeks to achieve a degree of acetylation where the chitosan is soluble (Roberts, 1992).

Treatment with 50% (w/v) NaOH is common but on a large scale this procedure is costly and has disadvantages with respect to controlling the reaction. CDA enzymes could help to lower costs and increase the sustainability of the deacetylation process (Younes *et al.*, 2016). CDA's have been studied for over 20 years (Kafetzopoulos, Martinou and Bouriotis, 1993), and their activities on chitin oligosaccharides have been studied to elucidate different deacetylation patterns, making for a precise and controllable reaction (Aranda-Martinez *et al.*, 2018; Hembach, Cord-Landwehr and Moerschbacher, 2017). However, it is not evident that these enzymes work on chitins with higher degrees of polymerisation as the hydrogen bonding throughout the chitin sheets may be too strong to break apart (Jaworska and Roberts, 2016). The ability to swell the chitin in a solution that is not going to hinder the enzyme action could be massively beneficial for developing an enzymic deacetylation step for high molecular weight chitosan. Alternatively lower crystallinity of the chitin would also facilitate swelling.

It is important to note that the homogenous and heterogenous reactions are not differentiated by their NaOH concentrations but mainly by their temperature and agitation conditions. Primarily homogenous deacetylation involves low temperature steps with NaOH to allow the NaOH to disrupt the extensive hydrogen bonding, permitting chitin chains to swell (Kurita, 2001).

Homogenous deacetylation is interesting from a practical perspective as it is claimed that it can produce water soluble chitosans (Kurita *et al.*, 1989). However, this homogenous approach does take significantly more time to conduct. Hence heterogenous deacetylation is more widely used for its quicker turnaround.

2.1.3.1 Insect chitosan production studies

Chitosan has been produced from a range of different insects (Ai *et al.*, 2008; Jing *et al.*, 2007; Hahn *et al.*, 2020; Nemtsev *et al.*, 2004). For degree of acetylation measurements, titration and FTIR are the most common. Deacetylation has been performed homogenously and heterogeneously, usually at concentrations of at least 10 M NaOH. A wide range of different DDAs from 34 up to 98% have been measured (Ai *et al.*, 2008; Jing *et al.*, 2007; Hahn *et al.*, 2020; Nemtsev *et al.*, 2004). However, ^{1}H NMR has not been used to measure the DA of insect derived chitosan.

2.1.4 MW characterisation

2.1.4.1 Chitosan molecular weight determination

Chitosan's properties and applications are primarily dependent upon the degree of acetylation (DA) and molecular weight (MW). During the procedures for extraction of chitin and production of chitosan, there is likely to be variation in the MW and DA both within and between samples. Uncharacterised chitosans used in studies can lead to unreproducible studies. Therefore, it is important to characterise the MW to be able to fully understand and reproduce studies on the properties of chitosan. There are several reported ways to determine the average MW of chitosans. Firstly, through intrinsic viscometric data and the Mark-Houwink equation (Kasaai, 2007):

$$[n] = KM^{\alpha}$$

where [n] = intrinsic viscosity, M = molar mass and the constants K and α are specific to a certain solvent and temperature.

The certificates of analysis of some commercial chitosans provide a dynamic viscosity based measurement in centipoise (cps). Typically along with reporting a centipoise value the certificate of analysis will provide the concentration and solvent used, and an estimation of MW. This is most commonly 1% (w/v) in 1% acetic acid.

Secondly, through light scattering, where the radius of gyration is measured. This is a more precise (and expensive) method which will often employ separation instruments such as gel permeation chromatography, size exclusion chromatography (GPC/SEC) or asymmetric flow field flow fractionation (AF4) combined with light scattering, a refractometer and a a

viscometer to provide accurate MWs in kDa (Brugnerotto *et al.*, 2001b; González-Espinosa *et al.*, 2019).

It is important to note, that in most cases it is likely that labs lack the expensive equipment necessary to determine the polymers MW and distribution. Low-cost, precise solutions to determine the MW are therefore needed. Viscometry is a low cost option for MW characterisation of polymers, which is regularly used in the literature. However, even then the precise temperature controlled baths can amount to significant costs.

2.1.4.2 Solubility and Preparation

In solution chitosan behaves as a polyelectrolyte as the free amine groups become positively charged when the polymer is dissolved in a dilute acidic medium (Rinaudo, 2006). However, it is important to note how crucial the preparations detail are to its behaviour. So, for example, after drying a chlorhydrate salt of chitosan, the chitosan will readily dissolve in water (Rinaudo, 2006). Chitosan has also been reported to be hygroscopic which means that thorough dehydration is required for accurate weighing. This means that great attention to detail is needed during the processing and preparation of chitosan, during its analysis and when reporting methods in scientific reports.

As a naturally derived cationic polyelectrolyte, the charges along the chain mean that chitosan in solution can have different chemical interactions with other chains and the solvent molecules. This could perturb viscosity measurements due to the formation of aggregates. Therefore, a salt is needed in the solution to help in minimising the interactions between chains and prevent providing misleading results. Precise use is necessary as the salt concentration and pH have been demonstrated to affect the MW determination (Brugnerotto *et al.*, 2001b; Lamarque *et al.*, 2005; Rinaudo, Milas and Le Dung, 1993). Several different acid/salt combinations have been used to study chitosan's rheological properties. Acetic acid and hydrochloric acid are most commonly used along with salts including sodium acetate, sodium chloride and ammonium acetate. Lamarque *et al.* (2005) and Czechowska-Biskup *et al.* (2018) used 0.2 M acetic acid and 0.15 M ammonium acetate. Brugnerotto *et al.* (2001b) and González-Espinosa *et al.* (2019) instead chose to focus on an acetic acid 0.3 M: 0.2 M sodium acetate as a solvent.

Aggregations, however, are still reported despite the use of salts, which led to the conclusion that other interactions aside from hydrogen bonding are leading the interactions between chains. It is now understood that this may be due to hydrophobic interactions (Philippova *et al.*, 2001; Philippova and Korchagina, 2012). Filtration has proven a solution to these aggregation issues. Using 0.45 and 0.2 µm filters has been shown to be extremely important (Brugnerotto *et al.*, 2001b; Czechowska-Biskup *et al.*, 2018; González-Espinosa *et al.*, 2019). On the other hand, for high molecular weights the loss of sample has been acknowledged to be a problem during filtration which may alter the polymer concentration considerably.

2.1.4.3 Chitosan specifics of viscometric studies

There is a wealth of data available from studies on chitosans that provide Mark-Houwink constants which facilitate the use of viscometry for determining MWs of chitosans (Table 2). These are normally specific for a certain solvent, polymer, temperature and pH of a system. There is a need to follow certain parameters carefully. Therefore following on from Aim 2, Objective 2 to develop an affordable method for measuring molecular weight of chitosans in the laboratory, there was sub-Objective 2.2.1 which required calibration of the specific system developed. This would further assist in achieving Aim 2, Objective 3 to characterise the molecular weights of chitosans.

Table 2 – Experimentally derived Mark-Houwink constants (K and α) from studies at different temperatures with different solvent compositions. HAc = acetic acid, NaAc = sodium acetate, AmmAc = ammonium acetate, NaCl = sodium chloride, DA = degree of acetylation, MW = molecular weight.

Solvent	**Temp (^0C)**	**pH**	**K ($mL\ g^{-1}$)**	**α**		
0.1 M HAc/0.2 M NaCl	25		0.00181	0.93	Roberts and Domszy, 1982	Rinaudo, 2006
0.1 M HAc/0.02 M NaCl	25		0.00304	1.26	Roberts and Domszy, 1982	
0.2 M HAc/0.1 M NaAc/4 M Urea	25		0.0893	0.71	Lee, 1974	
0.3 M HAc/0.2 M NaAc	25		0.082	0.76	Rinaudo *et al.*, 1993	
0.3 M HAc/0.2 M NaAc	25		0.079	0.796	Brugnerotto *et al.*, 2001	
0.02 M acetate buffer/0.1 M NaCl	25		0.0843	0.92	Berth and Dautzenberg, 2002	
0.02 M HAc/0.1 M NaAc/0.1 M NaCl	20	4.5	0.559	0.58	Anthonsen *et al.*, 1993	Kasaai, 2007
0.5 M HAc/0.5 M NaAc	25	4.7	0.199	0.59	Yomota *et al.*, 1993	
0.3 M HAc/0.2 M NaAc	25	4.6	0.082	0.76	Rinaudo *et al.*, 1993	
0.3 M HAc/0.2 M NaAc	25	4.6	0.076	0.76	Rinaudo *et al.*, 1993	
0.3 M HAc/0.2 M NaAc	25	4.6	0.074	0.76	Rinaudo *et al.*, 1993	
0.02 M HAc/0.1 M NaAc/0.1 M NaCl	20	4.5	0.0585	0.78	Anthonsen *et al.*, 1993	
0.25 M HAc/0.25 M NaAc	25	4.7	0.0157	0.79	Kasaai, 2000	
0.2 M HAc/0.1 M NaAc	30	4.4	0.0168	0.81	Wang *et al.*, 1991	
2% HAc/0.2 M NaAc	25	4.5	0.0138	0.85	Gamzazade *et al.*, 1985	
0.2 M HAc/0.1 M NaAc	30	4.4	0.00659	0.88	Wang *et al.*, 1991	
0.1 M HAc/0.2 M NaCl	25	2.8	0.00181	0.93	Roberts and Domszy, 1982	
0.2 M HAc/0.1 M NaAc	30	4.4	0.00142	0.96	Wang *et al.*, 1991	
0.33 M HAc/0.3 M NaCl	21	4.7	0.00341	1.02	Pogodina *et al.*, 1986	
0.02 M HAc/0.1 M NaAc/0.1 M NaCl	20	4.5	0.00218	1.06	Anthonsen *et al.*, 1993	
0.2 M HAc/0.1 M NaAc	30	4.4	0.000104	1.12	Wang *et al.*, 1991	
0.1 M HAc/0.02 M NaCl	25	2.9	0.0000304	1.26	Roberts and Domszy, 1982	
0.2 M HAc/0.1 M NaCl/4 M Urea	20	2.6	0.0893	0.71	Lee, 1974	
1% HAc	30	2.8	0.00474	0.72	Rao, 1993	
0.2 M HAc/0.2 M NaAc	25	4.3		1.14	Errington *et al.*, 1993	
0.17 M HAc/0.47 M NaCl	25	2.5	1.115	0.147	Berkovich *et al.*, 1980	
0.3 M HAc/0.2 M NaAc	35	4.5	0.0000229	1	González-Espinosa *et al.*, 2019	
0.3 M HAc/0.2 M NaAc	35	4.5	0.0000225	1	González-Espinosa *et al.*, 2019	
0.3 M HAc/0.2 M NaAc	35	4.5	0.0002632	0.83	González-Espinosa *et al.*, 2019	
0.3 M HAc/0.2 M NaAc	35	4.5	0.000695	0.73	González-Espinosa *et al.*, 2019	
0.3 M HAc/0.2 M NaAc	35	4.5	0.0004908	0.78	González-Espinosa *et al.*, 2019	
0.3 M HAc/0.2 M NaAc	35	4.5	0.0003397	0.81	González-Espinosa *et al.*, 2019	
0.2 M HAc/0.15 M AmmAc	25		0.0428	0.773	Czechowska-Biskup *et al.*, 2018	
0.2 M HAc/0.15 M AmmAc	25		0.0364	0.78	Czechowska-Biskup *et al.*, 2018	
0.2 M HAc/0.15 M AmmAc	25		0.095	0.75	Czechowska-Biskup *et al.*, 2018	

2.1.5 Viscometry background

Rheology is a scientific field of physics which encompasses the deformation of materials and their flow behaviour. Viscosity measurements are taken to help further understand the rheological properties of a material. Viscosity can be calculated from the shear stress divided by the shear rate through the rearrangement of Newton's Law (Kulicke and Clasen, 2004):

Viscosity = Shear stress / shear rate

Shear stress describes the behaviour of a liquid when an external force is applied to it. So, it is the force applied per unit area to a liquid, measured in N m^{-2} or Pa (Kulicke and Clasen, 2004). Shear rate is the velocity introduced from the applied force divided by the distance between the layer where the force has been applied and the stationary liquid layer; this gives the units s^{-1} (Kulicke and Clasen, 2004).

There are numerous ways to measure viscosity: Rotational, capillary, falling/rolling ball and pressurised viscometers (Kulicke and Clasen, 2004). In the literature, chitosan MW determination is commonly carried out in capillary viscometers (Figure 10) (Czechowska-Biskup *et al.*, 2018; Jing *et al.*, 2007; Nemtsev *et al.*, 2004; Rinaudo, Milas and Le Dung, 1993). Capillary viscometers have a sole force acting on the liquid (gravity). Kinematic viscosity capillary constants are provided by the manufacturer. Different sized capillaries allow different polymer sizes in different concentrations to be used, although typically one size is used for all polymer samples with a series of different dilutions.

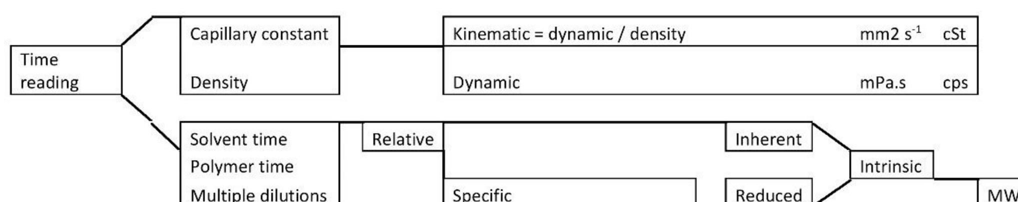


Figure 10 – Flow diagram for interpreting viscosity measurements and viscosity terms from flow readings to interpreting molecular weight (MW) through Mark-Houwink and how flow readings can be interpreted to provide centistokes (cSt, mm^2 s^{-1}) and centipoise (cps, mPa.s) values.

Viscometry measurements can be affected by several parameters: the way the fluid behaves itself (concentration, molar mass, solubility, chemical structure, branching), the external force being applied (gravity, density) and the ambient conditions (temperature, pH, solvent) (Kulicke and Clasen, 2004). Therefore it is important to control the ambient conditions as much as possible to provide accurate measurements for comparison.

2.1.6 Dilute solution viscometry

Polymer solutions typically show non-Newtonian behaviour. This means that changing the forces acting on the solutions results in different viscosities (shear dependent) (Kulicke and Clasen, 2004). Therefore, Newtonian solutions would have the same viscosity regardless of the shear rate (shear independent). However, when a polymer solution is diluted, it starts to show Newtonian behaviour. This region of dilution is sometimes referred to as a Newtonian plateau (Kulicke and Clasen, 2004).

Dilute solution viscometry is used to identify the intrinsic viscosity of a polymer, which can then be used to determine the molecular weight (Kulicke and Clasen, 2004). This is important in polymer chemistry as it gives an indication of polymer quality. Variation in polymer quality can result in variation of properties and applications.

The concentration at which polymer molecules begin to interact with others is defined as the critical concentration. The critical concentration is where the flow behaviour changes and individual polymer molecules begin to interact with one another rather than interacting with the solvent molecules meaning it is no longer dilute (Kulicke and Clasen, 2004). For dilute solution viscometry the concentration of the polymer needs to be below the critical concentration, aiming for an ideal dilute solution. This is so that when calculating the reduced viscosity from the specific viscosity, using Einstein's work on polymer coils, the higher powers of the Taylor series used to describe viscosity enhancing properties of a polymer coil become unnecessary and the equation simplifies considerably (Hughes, 1954). The ideal dilute solution requires a polymer solution to have no shear. Therefore, dilute solutions are used to reduce the shear acting on the solution to a minimum (Kulicke and Clasen, 2004). Measurements are taken on a series of dilutions for each polymer then the relative viscosity (Nr) is calculated for each dilution. This is the viscosity of the polymer solution (n) divided by the viscosity of the solvent system (ns)(Kulicke and Clasen, 2004):

$$Nr = n/ns$$

From the relative viscosity the inherent viscosity (Ninh) can be calculated with the concentration (c) and natural logarithm (Ln)(Kulicke and Clasen, 2004):

$$Ninh = Ln(Nr/c)$$

The specific viscosity (Nsp) is defined through subtracting the solvent flow time (ns) from the polymer sample (n) and dividing it by the solvent time (ns). So this viscosity is using the

additional flow time caused by the polymer over the flow time from the solvent instead of comparing the flow times as a ratio as is the relative viscosity. This can be simplified to the relative viscosity (Nr) value minus one (Kulicke and Clasen, 2004).

$$Nsp = np/ns = (n - ns)/ns = Nr - 1$$

From the specific viscosity (Nsp) the reduced viscosity (Nred) can be calculated. From Einstein's work on polymer coils, the concentration of the polymer can be isolated as an important factor (Kulicke and Clasen, 2004).

$$Nred = Nsp/c = 2.5/density$$

Therefore the reduced viscosity (Nred) takes concentration (c) into account. This then facilitates a linear plot which is extrapolated to x = 0 for the intrinsic viscosity [n].

2.1.7 Asymmetric flow field flow fractionation background

Asymmetric flow field flow fractionation (AF4) is a separation method used for characterisation of a wide range of different particles and macromolecules in a variety of applications. It is a useful alternative to SEC systems because there is no packing or a stationary phase which means a more reliable elution of larger molecules is possible (Cölfun and Antonietti, 2000). Often these systems are coupled with multi angle light scattering (MALS) and refractive index (RI) detectors for calculating molar mass and its distribution.

Field flow fractionation has a sample in carrier flow in a narrow channel with an external field applied to cause a size separation. Flow field flow fractionation has the addition of a cross flow. For AF4, the asymmetry is provided by the presence of an impermeable wall on one side of the channel and a semi permeable accumulation membrane on the opposite side allowing solvent to exit the channel (Figure 11) (Cölfun and Antonietti, 2000).

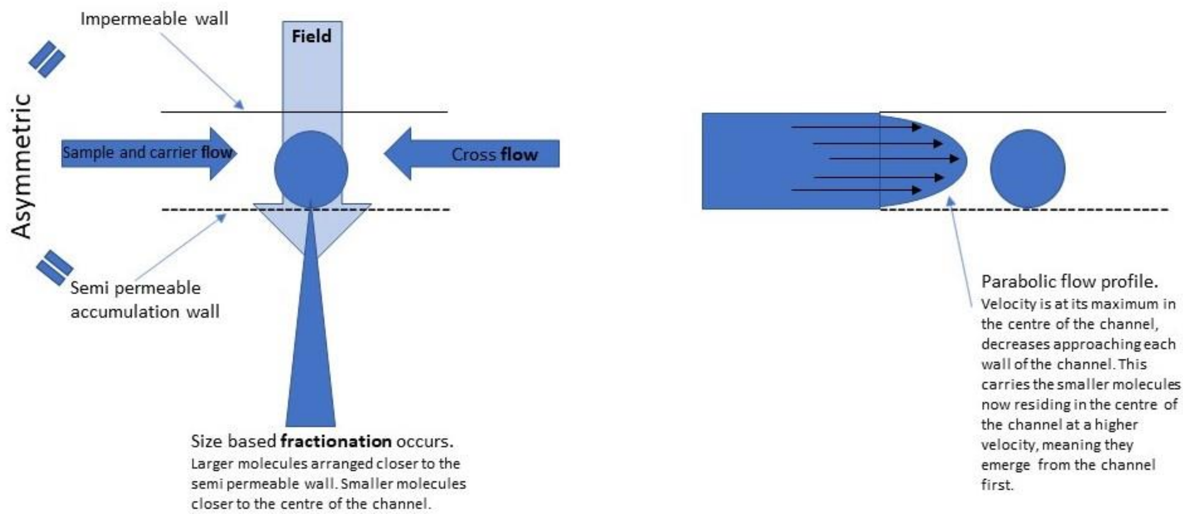


Figure 11 – The forces used in asymmetric flow field flow fractionation to separate the sample based on particle size.

AF4 is advantageous over symmetric F4 as it has the ability to focus the sample before eluting to reduce zone broadening issues (Cölfun and Antonietti, 2000). Compared with SEC, there is usually no filtration prior to input which allows identification of aggregates and prevents the loss of high MW samples. The primary disadvantage for AF4 relating to chitosan analysis has been due to potential interaction with the accumulation membrane (González-Espinosa *et al.*, 2019).

Chitosan molecular weight analysis has received a lot of attention through SEC/GPC analysis. But alternative methods including AF4 have been gaining more interest. Unlike SEC, AF4 has been able to distinguish aggregations in samples which makes it interesting for understanding the way the polymer is behaving in solution (González-Espinosa *et al.*, 2019; Kang *et al.*, 2021). Insect derived chitosan has not been analysed directly for its MW distribution.

2.1.8 Aims and objectives

The focus for this chapter was to extract and produce chitosan from black soldier fly prepupae giving an understanding of the quantity available from the life stage and to assess a green solvent method against the conventional chemical method. It became clear that a molecular weight analysis method was needed therefore a second objective was to establish a viscometry method which could be used to compare chitosan samples. Finally the degree of acetylation of chitosan samples would need to be characterised. Therefore chitosan

characteristics and their impact on antimicrobial activity could be investigated in later chapters.

The **aims** and objectives for this chapter are further summarised:

1 Extract chitin from the black soldier fly

1.1 Extract chitin comparing two chemical based methods on a small scale

1.2 Increase the scale of the extraction to provide chitosan on a gram scale.

2 Deacetylate chitin and characterise the chitosan

2.1 Degree of acetylation characterisation

2.2 Develop an affordable method for measuring molecular weight of chitosans in the lab

2.2.1 Calibration of the specific system developed

2.3 Characterise the molecular weight of chitosans

2.2 Materials and methods

2.2.1 General materials

2.2.1.1 Chemicals and consumables:

D-glucosamine (>98% percent purity) - Fisher Scientific, UK

N-acetyl-β-D-glucosamine (>98% assay percent range) - Fisher Scientific, UK

2 mL microcentrifuge tubes, Eppendorf - Fisher Scientific, UK

Whatman 540 15 cm filters - Fisher Scientific, UK

Choline chloride - Fisher Scientific, UK

Urea - Fisher Scientific, UK

Polypropylene containers 1 L - Cole Parmer, UK

Sodium hydroxide pellets - Fisher Scientific, UK

Glacial acetic acid – Fisher Scientific, UK

6 M hydrochloric acid – Sigma Aldrich, Germany

Ammonium acetate – Sigma Aldrich, Germany

Sodium acetate - Fisher Scientific, UK

Deuterium oxide (D_20) NMR grade Fisher Scientific, UK

Deuterium chloride (DCl) NMR grade - Fisher Scientific, UK

Deionised water (ddH_2O) grade 18.2 Ω.

Black soldier fly prepupae - Fera Science Ltd, UK.

SpecialIngredients food grade sodium alginate - Amazon.co.uk

Chitosans – Glentham Life Sciences, UK

3-(trimethylsilyl)propionate-d_4 (TSP) - Fisher Scientific, UK

2.2.1.2 Equipment

IKA A11 analytical mill

Bruker Ultrashield Plus 500 MHz NMR spectrometer

Bibby HB502 magnetic stirrer

Eppendorf MiniSpin microcentrifuge

Memmert laboratory oven

Fisherbrand whirlmixer vortex – Fisher Scientific, UK

Balance (0.1 mg) – Sartorius, Germany

Edwards SuperModulyo Freeze Dryer

Techne DB-2P Dri-Block Heater

Balance (0.01 g) – Sartorius, Germany

Ubbelohde Viscometer 525 10/I (capillary size 0.58 mm) - Xylem Analytics Germany GmbH

SevenEasy pH meter, Mettler Toledo

Postnova's asymmetric flow field flow fractionation coupled with multiangle light scattering and refractive index (AF4-MALS-RI)

Calibrated laboratory thermometer

2.2.2 Extraction

2.2.2.1 Deep eutectic solvents (DES) vs conventional extraction method

Chemical reagents were purchased from Fisher Scientific, UK. Black soldier fly prepupae (BSFP) from Fera Science Ltd were frozen at –80 °C then freeze dried for 24 hours, ground in an IKA A11 analytical mill, washed and skimmed with water in excess. Once dried, 50 mg of BSFP powder was weighed into 2 mL microcentrifuge tubes.

For the conventional extraction the method from Caligiani *et al.* (2018) was followed with some minor changes. 1 M NaOH was applied for two hours at 80 °C (1 g in 20 mL) on a preheated dry block with agitation. The solid was then washed extensively with water, followed by acetone and then dried. 2 M HCl was added (1 g in 20 mL) at room temperature overnight with agitation. The wash steps were then repeated.

For DES extractions, the method of Zhou *et al.* (2019) was followed. Choline chloride and urea in a 1:2 molar ratio were combined in an Erlenmeyer flask on a dry heat block to form

the DES. BSFP powder had DES applied (1 g in 20 mL). This was vortexed thoroughly and placed on a dry heat block with four hour incubation at 80 °C and agitation. Once incubation was completed warm water was added, the mixture was centrifuged and the precipitate washed extensively with water. The precipitate was then washed with acetone and allowed to air dry.

For estimated yields, the gravimetric weights of material were measured before commencing the treatment and then weighed post treatment to calculate remaining mass (percentage yield). The DES extraction had six replicates, the traditional had five. Statistical analysis consisted of Kolmogorov-Smirnov normality tests, Levene's variance test and t-tests which were performed using IBM SPSS Statistics software.

2.2.2.2 Prepupae to chitosan via conventional method on a gram scale

Whole prepupae of the BSF (*Hermetia illucens*) were frozen at –80 ^{o}C and lyophilised for 24 hours and subsequently weighed. The prepupae were then milled using an IKA A11 analytical mill at 50/60 Hz, washed in ddH_2O, frozen at –80 ^{o}C and lyophilised for 24 hours, then weighed. To remove fats the powdered BSF were treated with three 500 mL washes of hexane, air dried in a fume hood and weighed again. To remove protein the dried powder was then treated with 1 M NaOH (26 g in 1 L) at 50 ^{o}C for two hours, before being washed with ddH_2O, neutralised, frozen at –80 ^{o}C, lyophilised for 24 hours and weighed. To remove minerals the remaining powder was treated with 2 M HCl (13 g in 1 L) for 24 hours at room temperature with agitation. After 24 hours the solid remaining was again washed, neutralised, frozen at –80 ^{o}C, lyophilised for 24 hours and weighed.

Deacetylation of the extracted chitin was performed under homogenous conditions using 12.5 M NaOH on cycles at 60 ^{o}C for 20 hours (30 g in 1 L), then tested for solubility in mild acidic conditions. The alkali treatment cycle was repeated a further two times. This achieved a chitosan sample that remained solid at alkaline and neutral pH but fully dissolved at mild acidic pH (1% acetic acid). The chitosan was first precipitated and washed to neutral pH then dissolved in dilute acetic acid and filtered through a Whatman 540 filter. This was again precipitated at neutral pH, frozen at –80 ^{o}C and lyophilised for 24 hours, and weighed to provide a yield of chitosan. This chitosan, along with commercially available chitosans, were subject to further analysis for characterisation.

Chitosan yield from chitin was calculated as:

Chitosan yield = (chitosan mass (g)/chitin mass (g)) x 100

2.2.3 Deacetylation reactions

2.2.3.1 Deacetylation 1

Samples of commercially available chitin from Glentham Life Sciences were incubated at room temperature for five days in 5 M NaOH before being subjected to 80 ^{o}C with agitation on a dry heat block for between 0 to 72 hours. Several samples were then neutralised with an equimolar volume of 6 M HCl (A). Several samples were not neutralised (B). Alongside deacetylation, several commercial chitin (C) and chitosan (D) samples were subjected to 3 M HCl at 80 ^{o}C for varying hours (as stated below) then neutralised with 5 M NaOH.

2.2.3.2 Deacetylation 2

Chitin samples were heated to 70 ^{o}C in the presence of the following conditions:

1. 5 M NaOH for 24 h
2. 7.5 M NaOH for 24 h
3. 10 M NaOH for 6 h
4. 10 M NaOH for 24 h
5. Glentham Life Science medium molecular weight chitosan was used as a positive control.

Each of these samples were then neutralised and washed and dried, and dissolution in 0.6% (v/v) DCl/D_2O was attempted at a concentration of 6 g L^{-1}. Samples were run on ^{1}H NMR at 70 ^{o}C following the same experimental conditions as Lavertu *et al.* (2003). DDA was calculated according to the following:

DDA (%) = (H-1D/ (H-1D + [H-Ac/3]))*100

H-1D = integral of peak 1 (ppm ~5.3); H-Ac = Integral of peak 2 (ppm ~2.45)

To compare the dissolution, a purity value was calculated based on the signals acquired in comparison to a fully soluble chitosan (sample 5 from above). This was calculated from the same peaks used to calculate the DDA and provided a % value.

Integral of peak 1 (~5.3 ppm) + (Integral of peak 2 (~2.45 ppm) divided by 3) = total value

Purity value (%) = (Total value for a given sample/total value for chitosan control)*100

2.2.3.3 Deacetylation 3 – BSF chitin

BSF derived chitin was deacetylated as previously stated in Section 2.2.2.2 and purified based on its solubility in 1% acetic acid and precipitation when adjusted to mild alkali pH.

2.2.4. ^{1}H NMR for determination of binary heteropolysaccharide composition

2.2.4.1 M/G ratio of food grade alginate

Alginate is a binary polysaccharide which can be isolated from brown seaweed. Seaweed growth can be useful for blue biotechnology as earlier stated in Chapter 1, most importantly it requires no fresh water or land use compared with terrestrial plant sources and grows rapidly (Doi *et al.*, 2017). In addition, alginate is anionically charged which means it is oppositely charged to chitosan and they can form polyelectrolyte complexes together (Castel-Molieres *et al.*, 2018). Like the degree of acetylation affects the properties of chitosan (Aranaz *et al.*, 2009), the proportion of the different uronic acid sugars in alginate influences its properties (Aarstad *et al.*, 2012), and may impact its ability to interact with chitosan. Therefore in order to provide sufficient information to reproduce the experiments fully it is important to know the proportions of mannuronic and guluronic acid residues present in the alginate sample used.

Alginate composition was analysed by ^{1}H NMR by the methods of Grasdalen (1983) and ASTM (2012). Briefly, dry alginate powder was subject to a mild hydrolysis with HCl. Alginate (0.1% (w/v)) dissolved in water was brought to pH 5.6 with HCl and placed in a boiling water bath for an hour. The pH was adjusted to 3.8 and the solution was replaced back into the water bath for a further 30 minutes. The pH was then brought back to neutral and frozen at –80 ^{o}C and lyophilised for 24 hours, then prepared for ^{1}H NMR in D_2O at 10 g L^{-1}. ^{1}H NMR spectroscopy was ran on a Bruker UltraShield Plus 500 MHz spectrometer at 80 ^{o}C, 64 scans, 2 s relaxation, 90° proton pulse angle, 4.096 s acquisition, 1D pulse program at 20 Hz. The ^{1}H NMR spectrum for the food grade alginate sample is illustrated in Figure 12.

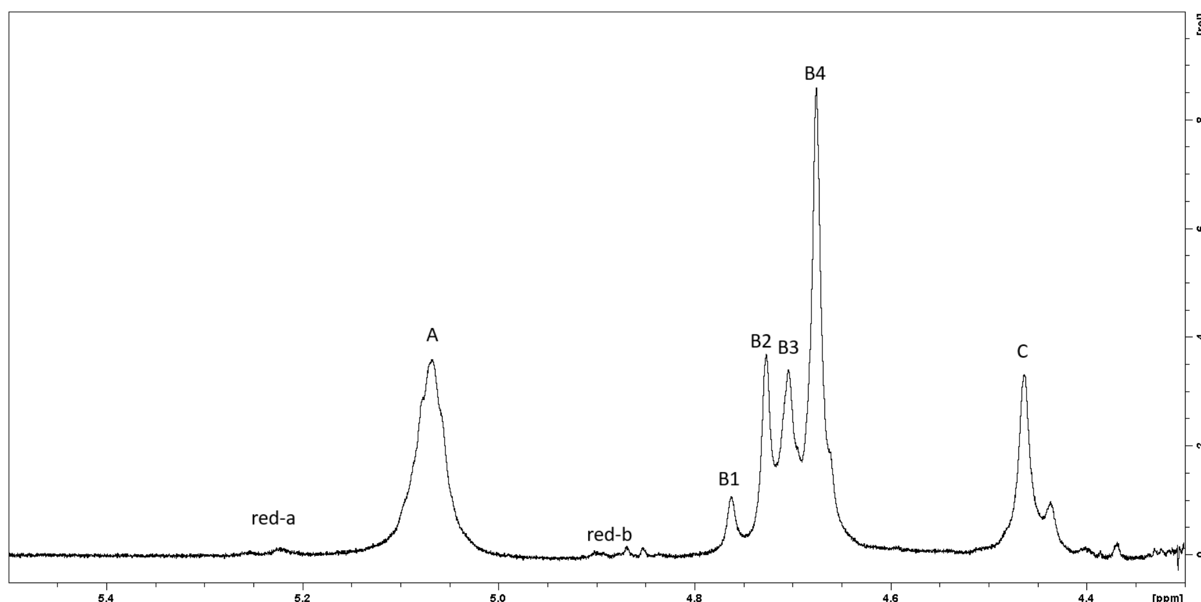


Figure 12 – ^{1}H NMR spectra for food grade alginate labelled for calculating alginate sequence and composition. Integrals for the peaks (red-a, A, red-b, B1, B2, B3, B4, C) of the ^{1}H NMR spectra were used to calculate the frequency (F_{XX}) with which certain sequences of alginate monomers (β-D-mannuronic acid = M, α-L-guluronic acid = G) occur within the structure of the alginate according to equations from ASTM (2012).

Integrals for the peaks (red-a, A, red-b, B1, B2, B3, B4, C, labelled in Figure 12 of the ^{1}H NMR spectra were used to calculate the frequency (F_{XX}) with which certain sequences of alginate monomers (G = guluronic acid, M = mannuronic acid) occur within the structure of the alginate according to equations from ASTM (2012).

$G = 0.5(A + C + 0.5(B1+B2+B3))$

$M = B4 + 0.5(B1+B2+B3)$

$GG = 0.5(A + C - 0.5(B1+B2+B3))$

$MG = GM = 0.5(B1+B2+B3)$

$MM = B4$

$GGM = MGG = (B1)0.5(B1+B2+B3)/(B1+B2)$

$MGM = (B2)0.5(B1+B2+B3)/(B1+B2)$

$GGG = GG - GGM$

$F_G = G/(M+G)$

$F_M = M/(M+G)$

$F_{GG} = GG/(M+G)$

$F_{MM} = MM/(M+G)$

$F_{GM} = F_{MG} = MG/(M+G)$

$F_{GGG} = GGG/(M+G)$

$F_{MGM} = MGM/(M+G)$

$F_{GGM} = F_{MGG} = GGM/(M+G)$

2.2.4.2 Measuring the degree of deacetylation of chitosan samples

Degree of deacetylation (DDA) of commercial and BSF derived chitosans was determined by solubilising samples in D_2O:DCl:TSP (3-(trimethylsilyl)propionate-d_4). TSP was used as the internal standard at a concentration of 1 mM. Chitosan was dissolved at a concentration of 6 g L^{-1} in 0.4% DCl: D_2O with TSP. ^{1}H NMR was ran following Lavertu *et al.* (2003)'s experimental setup at 70 ^oC. 64 scans, 8 s relaxation, 90° proton pulse angle, 2 s acquisition. Degree of deacetylation (DDA) was calculated according to equation 1 from Lavertu *et al.* (2003):

$$DDA (\%) = (H\text{-}1D / (H\text{-}1D + [H\text{-}Ac/3]))*100$$

H-1D = integral 1 (ppm ~5.3); H-Ac = Integral 2 (ppm~2.45)

2.2.5 MW characterisation

2.2.5.1 Materials

Chitosan samples were acquired from Glentham Life Sciences for comparison with the BSF produced chitosan. These included: ultra-low (UL) molecular weight (ULMW) (referred to as 5cps) and a newer batch (NBULMW)(referred to as NB 5cps); very-low (VLMW)(referred to as 10cps); medium (MMW); high (HMW); and Squid derived (Sq) chitosans. These were accompanied with certificate of analyses which provided estimations for characteristics (Table 3).

Table 3 – Properties reported on the certificates of analysis for commercially available chitosans used. DDA = degree of deacetylation, cps = centipoise, MW = molecular weight.

	ULMW (5cps)	NB ULMW (5cps)	VLMW (10cps)	MMW	HMW	Squid
DDA (%) (^{1}H NMR) (manufacturer)	90.27	94.07	94.83	90.21	90.99	93.77
DDA (%) (^{1}H NMR) (own study)	86.96	87.40	87.67	88.61		93.29
cps (1% in 1% acetic acid, 20 ^oC)	4.84	3.84	9.96	742.00	1080.00	75.40
Mw estimate (kDa)	20	20	30	1250	1500	580

An Ubbelohde Viscometer 525 10/I was purchased from Xylem Analytics Germany GmbH with a capillary size of 0.58 mm. Chemicals used included glacial acetic acid (HAc), sodium acetate (NaAc), ammonium acetate (AmmAc), acetone and deionised water (ddH_2O) grade 18.2 Ω.

2.2.5.2 Ideal dilute solution viscometry for viscometric average molecular weight

Chitosan has several methods that are employed for MW analysis which require expensive equipment. Viscometry was determined to be relatively cheap with respect to the viscometer, with the majority of the expense coming from a temperature controlled tank. Therefore an Ubbelohde viscometer was used in an adapted water tank and the temperature was monitored throughout experiments as depicted in Figure 13.

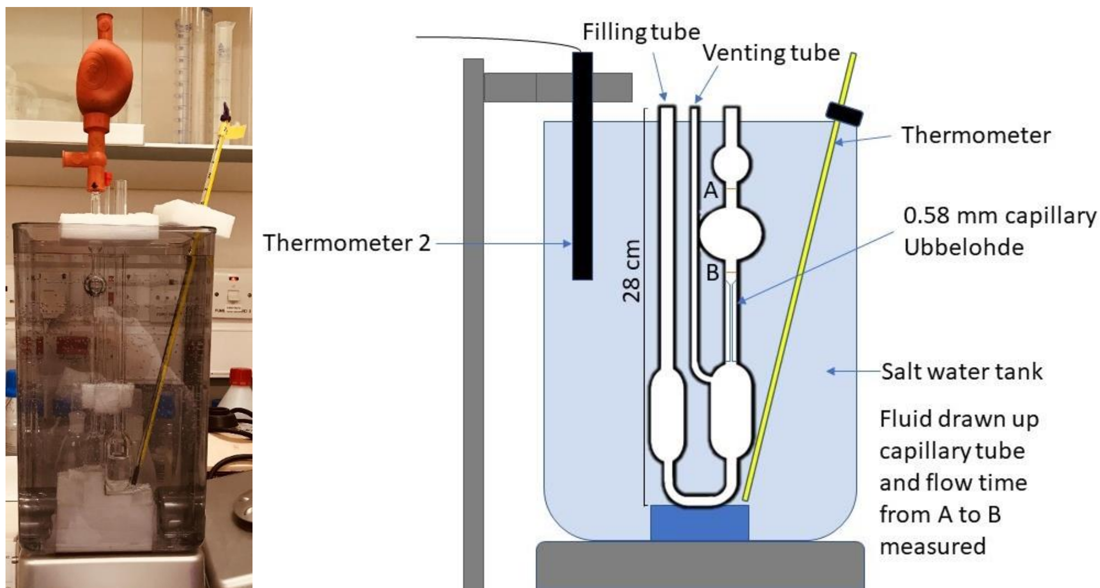


Figure 13 – Ubbelohde viscometer with a 0.58 mm capillary set up in a water tank with calibrated thermometers for monitoring temperature.

Following recommendations from Kulicke and Clasen (2004), viscometry was performed on dilute chitosan samples which produced 1.2-2.5 relative viscosity when compared to the buffer. The solvents used were 0.3 M HAc/0.2 M NaAc (pH 4.5) and 0.2 M HAc/ 0.15 M AmmAc (pH 4.5). Chitosan concentrations were varied for each sample to achieve Newtonian plateau in the 1.2 -2.5 relative viscosity regions. Chitosan samples were allowed to dissolve for 24 hours at room temperature before use. For each chitosan sample, at least five dilutions were measured to produce a trend that could be extrapolated to the y axis for intrinsic viscosity determination.

Temperature was monitored with calibrated thermometers throughout the measurements and ranged between 17.5-18.5 ^{0}C. For each sample measured, 15 mL of dilute sample was added to the viscometer. The sample was left for five minutes to acclimatise to the bath temperature and was then drawn up and allowed to flow once before measurements were taken. Five readings were taken with temperature readings taken immediately after commencement of each viscometry reading. For cleaning, the sample was decanted from the viscometer, which was then completely filled with solvent, decanted, followed by three washes with ddH_2O and three acetone washes and purged with nitrogen.

For determining the intrinsic viscosity the following equations from Kulicke and Clasen (2004) were used. The relative viscosity (Nr) is calculated for each dilution. This is the viscosity of the polymer solution (n) divided by the viscosity of the solvent system (ns):

$$Nr = n/ns$$

From the relative viscosity the inherent viscosity (Ninh) can be calculated with the concentration (c) and natural logarithm (Ln):

$$Ninh = Ln(Nr/c)$$

Then the specific viscosity (Nsp) is defined as:

$$Nsp = Nr - 1$$

From the specific viscosity (Nsp) the reduced viscosity (Nred) can be calculated which removes dependence on concentration (c):

$$Nred = Nsp/c$$

Plotting the concentrations of a polymer against reduced or inherent viscosity and extrapolating to x = 0 gives the intrinsic viscosity (Kulicke and Clasen, 2004). A plot of the same polymer with different intrinsic viscosities results in a graph from which the MW can be deduced through the Mark-Houwink equation:

$$[n] = KM^{\alpha}$$

The constants K and α are specific to a certain solvent and temperature (Kulicke and Clasen, 2004). M stands for molecular weight and [n] is the intrinsic viscosity. A plot of the same polymer with different MW's can elucidate the constants K and α. Log (MW) vs Log ([n]) where Log K = y intercept ; α = the gradient of the line of best fit (Kulicke and Clasen, 2004).

2.2.5.3 Asymmetric flow field flow fractionation coupled with multiangle light scattering and refractive index measurements for polymer molecular weight analysis

Three chitosan samples were analysed through Postnova's asymmetric flow field flow fractionation coupled with multiangle light scattering and refractive index (AF4-MALS-RI). The BSF chitosan sample extracted, 10cps and squid derived chitosans were used.

González-Espinosa *et al.* (2019) has previously examined the performance of AF4-MALS-RI for chitosan MW characterisation. González-Espinosa *et al.* (2019) recommended using acetate buffer at pH 3.7 to improve elution profiles. However, to draw on relative polymer measurements between methods, the same buffer as used for the viscometry component was chosen (0.3 M HAc; 0.2 M NaAc, pH 4.5). This eluent was filtered through 0.1 µm filter.

The samples were freeze dried as earlier described and posted to Postnova's laboratory in Germany for analysis using the following equipment and conditions:

- PN5300 Auto Injector, AF2000 Flow FFF system (AF4),
- AF2000 Analytical Channel (AF4), 350 µm thickness;
- NovaRC 10 kDa Membrane
- PN3211 UV – Absorbance, 254 nm;
- PN3621 MALS – static light scattering, 532 nm;
- PN3150 RI – Refractive index. Flow rate 0.5 mL min^{-1};
- Dn/dc =0.19 mL g^{-1}; 90° LS signal;
- 100 µl injection volume.
- Samples dissolved in eluent at a concentration of 2 g L^{-1}

Molar mass was calculated from MALS and RI data with a refractive index increment (dn/dc) of 0.19 mL g^{-1}.Calculations were based on a fit by Random Coil Model.

Number average molecular weight (Mn) = an arithmetic mean of the molecular fractions measured (Mn = n molar mass measurements / n).

Weight average molecular weight (Mw) = n molar mass measurements squared / n molar mass measurements.

Centrifugation average molecular weight (Mz) = n molar mass measurements cubed / n molar mass measurements squared.

2.3 Results

2.3.1 Extraction

2.3.1.1 Deep eutectic solvents vs conventional extraction method

DES and conventional mass yield data conformed to a normal distribution (Kolmogorov-Smirnov, P>0.05). The variances of the two samples could be considered equal (Levene's test, F = 4.041, P>0.05) and therefore a t-test assuming equal variances was conducted. There was a statistically significant difference in mean mass of chitin extracted from DES (mean = 59.17 ± 2.57 S.D.) and conventional extraction methods (mean = 10.68 ± 0.64 S.D.) (t-test, t = 40.794, df = 9, P<0.05) (Figure 14).

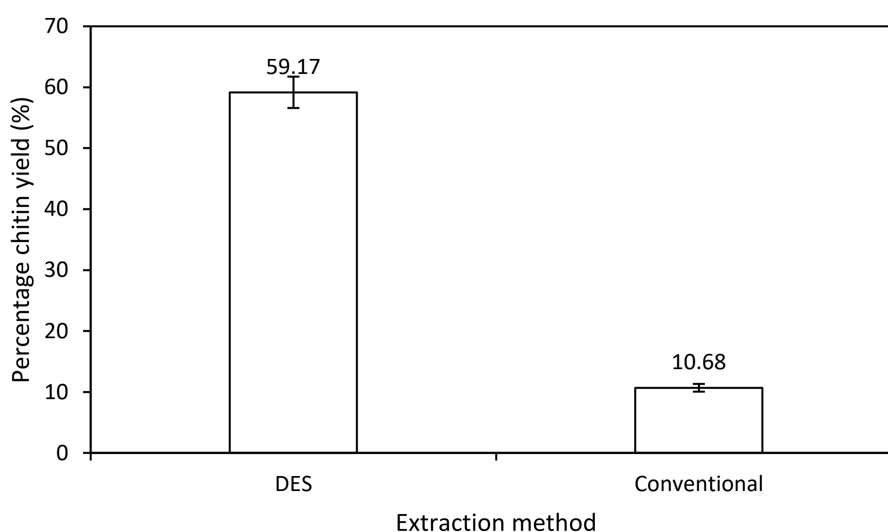


Figure 14 – Mean (± S.D.) percentage yields of chitin from conventional and deep eutectic solvent (DES) extraction procedures from black soldier fly prepupae. Conventional extraction consisted of 1 M NaOH and 2 M HCl. DES extraction consisted of choline chloride and urea in a 1:2 molar ratio.

2.3.2 Conventional extraction

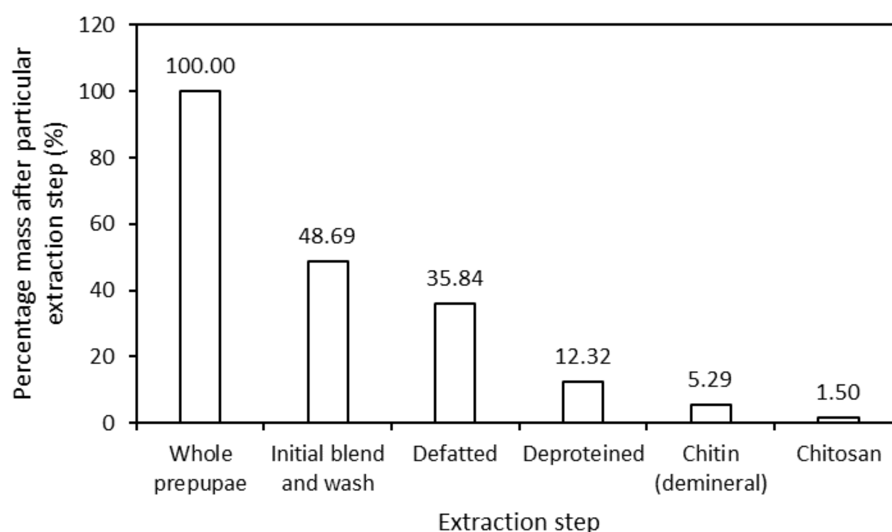


Figure 15 – The relative gravimetric masses (%) following each step of the conventional extraction at gram scale. Whole black soldier fly prepupae were blended in an analytical mill and washed with deionized water, defatted with hexane, deproteinated with 1 M NaOH and demineralised with 2 M HCl followed by deacetylation under homogenous conditions in 12.5 M NaOH.

Chitin was successfully extracted on a gram scale from whole BSF prepupae with a yield of 5.29% (Figure 15). This yield of chitin from the g scale was approximately half of what was obtained on a mg scale. This could be due to impurities remaining present in the small scale. For example, compared with the larger scale, the smaller scale extraction did not remove fats with hexane which could mean some remain present at the end of the extraction. It could also be due to loss of chitin sample during the larger scale extraction. The conditions may also have variable effects on chitin and cause degradation to different degrees. Purity analysis could be useful to confirm what has happened here.

2.3.4 ^{1}H NMR for determination of binary heteropolysaccharide composition

2.3.4.1.M/G ratio of alginate

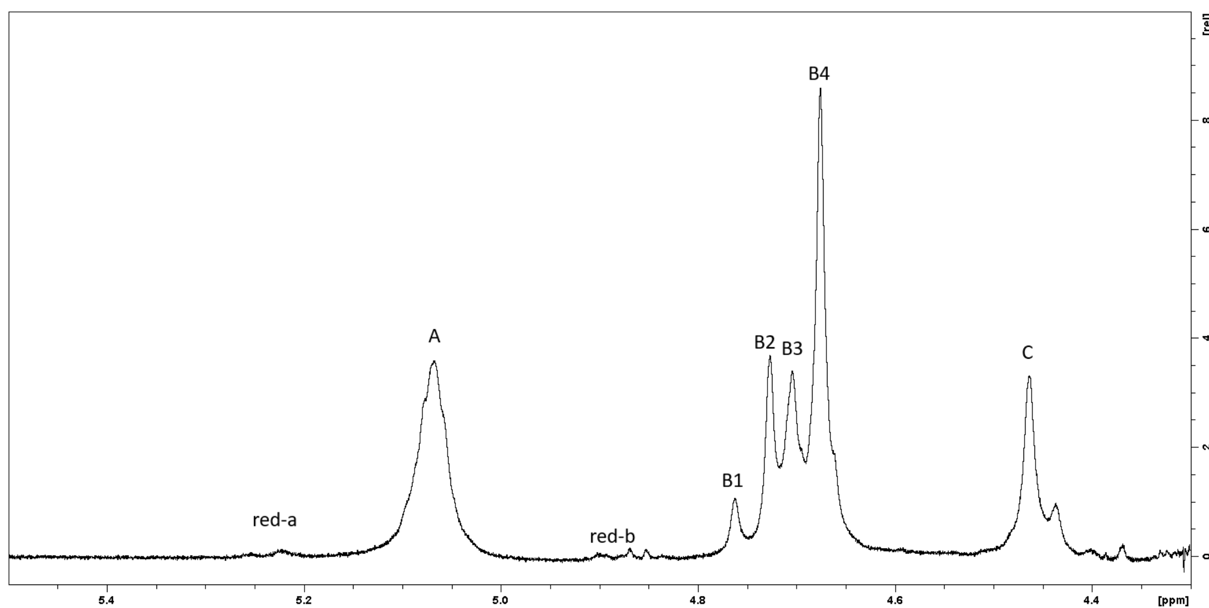


Figure 16 – ^{1}H NMR spectrum for food grade alginate (β-D-mannuronic acid (M) and α-L-guluronic acid (G) linked (1→4)). Integrals for the peaks (red-a, A, red-b, B1, B2, B3, B4, C) of the ^{1}H NMR spectra were used to calculate the frequency (Fxx) of certain sequences of alginate monomers (β-D-mannuronic acid = M, α-L-guluronic acid = G) occuring within the structure of the alginate according to equations from ASTM (2012) as described in section 2.2.4.1.

Table 4 – Frequencies (%) of β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues for food grade alginate determined by ^{1}H NMR according to ASTM (2012) and Grasdalen (1983).

Monomer sequence	**Occurrence of sequence (%)**
Fg	30.91
Fm	69.09
Fgg	9.61
Fmm	47.78
Fgm=Fmg	21.31
Fggg	4.84
Fmgm	16.54
Fggm=Fmgg	4.76

Figure 16 shows the ^{1}H NMR spectrum for food grade alginate. According to the equations from ASTM (2012) and as earlier described in section 2.2.4.1 the food grade alginate was determined to have a guluronic acid (G) monomer content of 30.9% and a mannuronic acid (M) content of 69.1% from the integrals of the ^{1}H NMR spectrum (Figure 16, Table 4). The

frequency of consecutive MM units was determined to be 47.78% while the frequency of GG units was determined to be 9.61%.

2.3.4.2 Chitosan DA

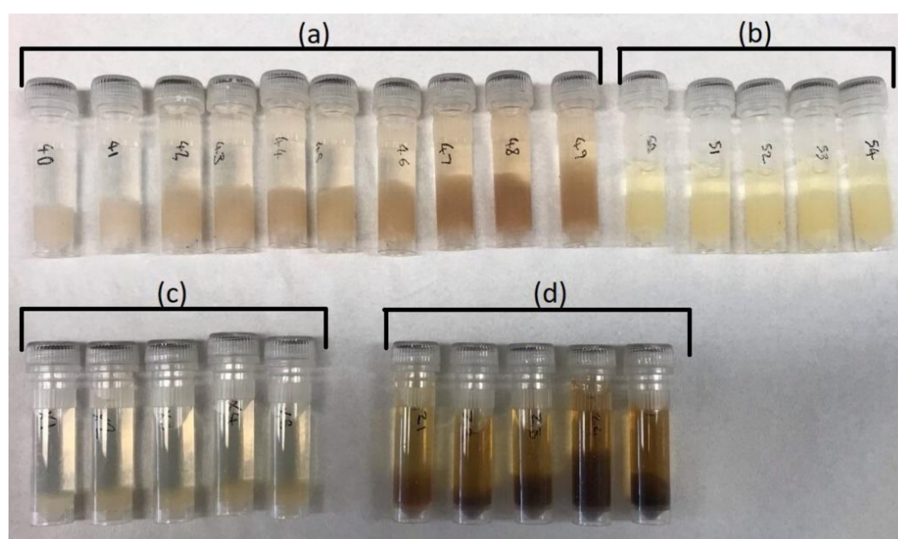


Figure 17 – Differently treated chitin and chitosans. a) 5 M NaOH 0 – 72 h and neutralised with HCl, depicting a colour scale as reaction conditions take effect. b) the same deacetylation conditions but not neutralised. c) chitin hydrolysis in 3 M HCl, neutralised with NaOH. d) chitosan hydrolysis in 3 M HCl and neutralised with NaOH. Increasing incubation time from left to right. None of the deacetylation reaction samples produced chitosan soluble in 1% acetic acid.

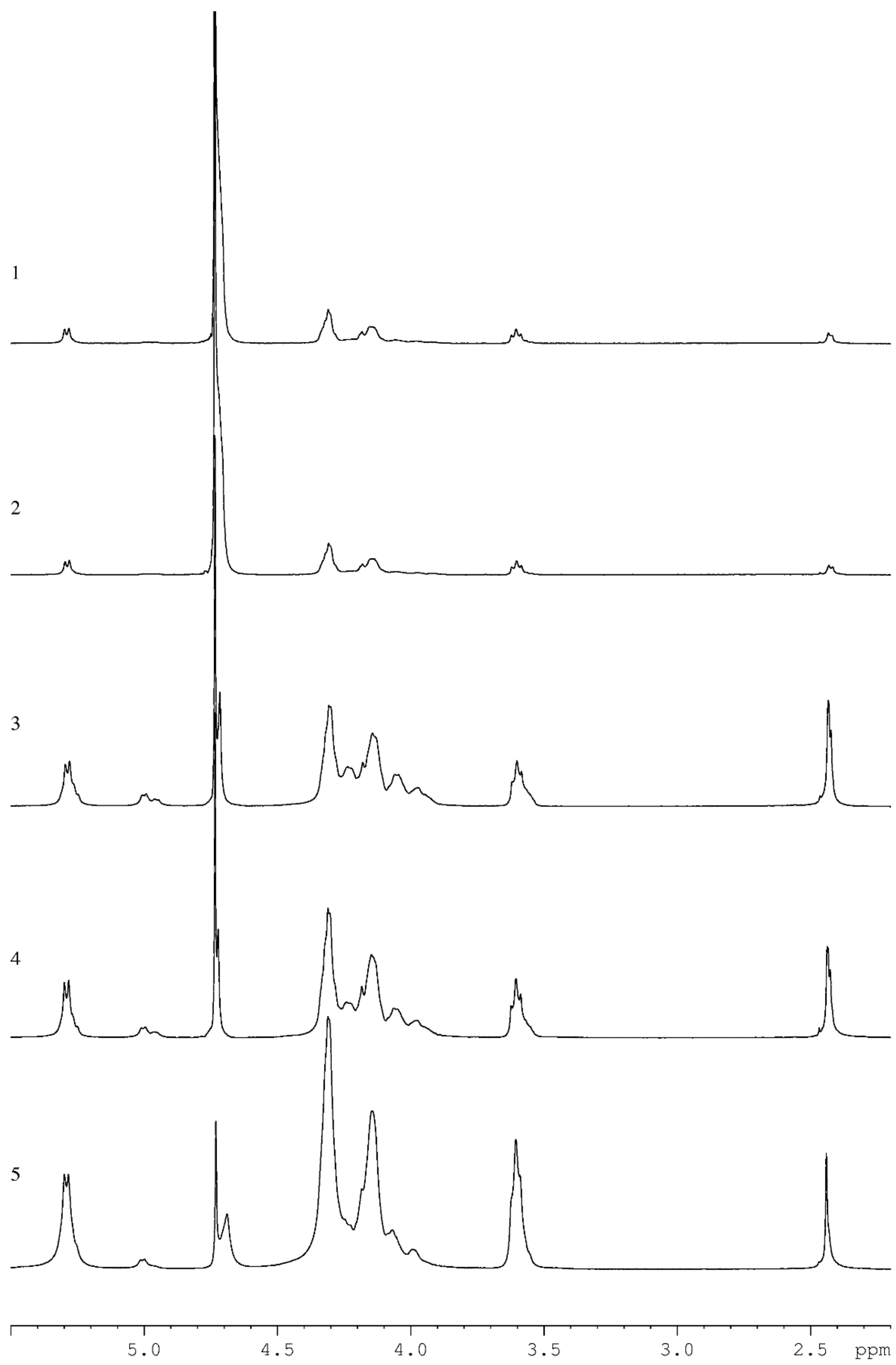

1
2
3
4
5
5.0
4.5
4.0
3.5
3.0
2.5
ppm

Figure 18 – ^{1}H NMR Spectra for differently deacetylated chitosans. Heating temperatures were 70 ^oC for all. 1. 5 M NaOH 24 hours, 2. 7.5 M NaOH 24 hours, 3. 10 M NaOH 6 hours, 4. 10 M NaOH 24 hours, 5. Glentham Life Science medium molecular weight chitosan standard. All at a concentration of 6 g L^{-1} in 0.6% DCl/D_2O. H1D = integral of the peak (ppm ~5.3) corresponding to the proton attach to the carbon atom labelled in Figure 9. HAc = integral of the peak (ppm ~2.45) corresponding to the acetyl protons. H1A = integral of the peak (ppm ~4.9) corresponding to the proton attached to the carbon atom labelled in the Figure 9. H26 = integral of the peaks (~3.8-4.2 ppm) corresponding to the monomer backbone protons.

Figure 17 shows the differences in colouration after different treatment times for deacetylation and hydrolysis reactions. Figure 18 is a depiction of differences in chitosan purity due to solubility difficulties. Purity could be estimated by direct comparison of integral values to the fully soluble standard. In the figure, it is illustrated by the sizes of the key peaks at 2.45 and 5.3 ppm, and the backbone peaks in the region 3.5-4.5 ppm, which if fully 'pure' would be similar sizes. The purity definition here actually more closely resembles the solubilisation efficacy for the reaction products.

Table 5 - Comparison of the degrees of deacetylation (DDA) and purity of chitins deacetylated with different NaOH concentrations and durations. Heating temperatures were 70 ^oC for all. 1. 5.0 M NaOH 24 hours, 2. 7.5 M NaOH 24 hours, 3. 10.0 M NaOH 6 hours, 4. 10.0 M NaOH 24 hours, 5. Glentham Life Science medium molecular weight chitosan standard. All at a concentration of 6 g L^{-1} in 0.6% DCl/D_2O. ^{1}H NMR spectra are illustrated in Figure 19.

Experiment	**NaOH (M)**	**Duration (h)**	**DDA (%)**	**Purity (%)**
1	5.0	24	83.62	9.48
2	7.5	24	81.38	9.36
3	10.0	6	70.51	58.29
4	10.0	24	75.35	53.99
5	Control	Control	88.61	100.00

Chitosan that was soluble in mild acidic media was successfully produced with 5.0 M, 7.5 M and 10.0 M NaOH and examined by ^{1}H NMR (Table 5). The yields of soluble chitosan were poor for the lower NaOH concentrations, but the lower concentrations achieved a higher

DDA than the highest concentration of NaOH. This highlights that even with homogenous reaction conditions there is likely to be variability in measured DA within one sample. Increasing the NaOH concentration may help deacetylate chitin to a more significant degree and provide a better yield.

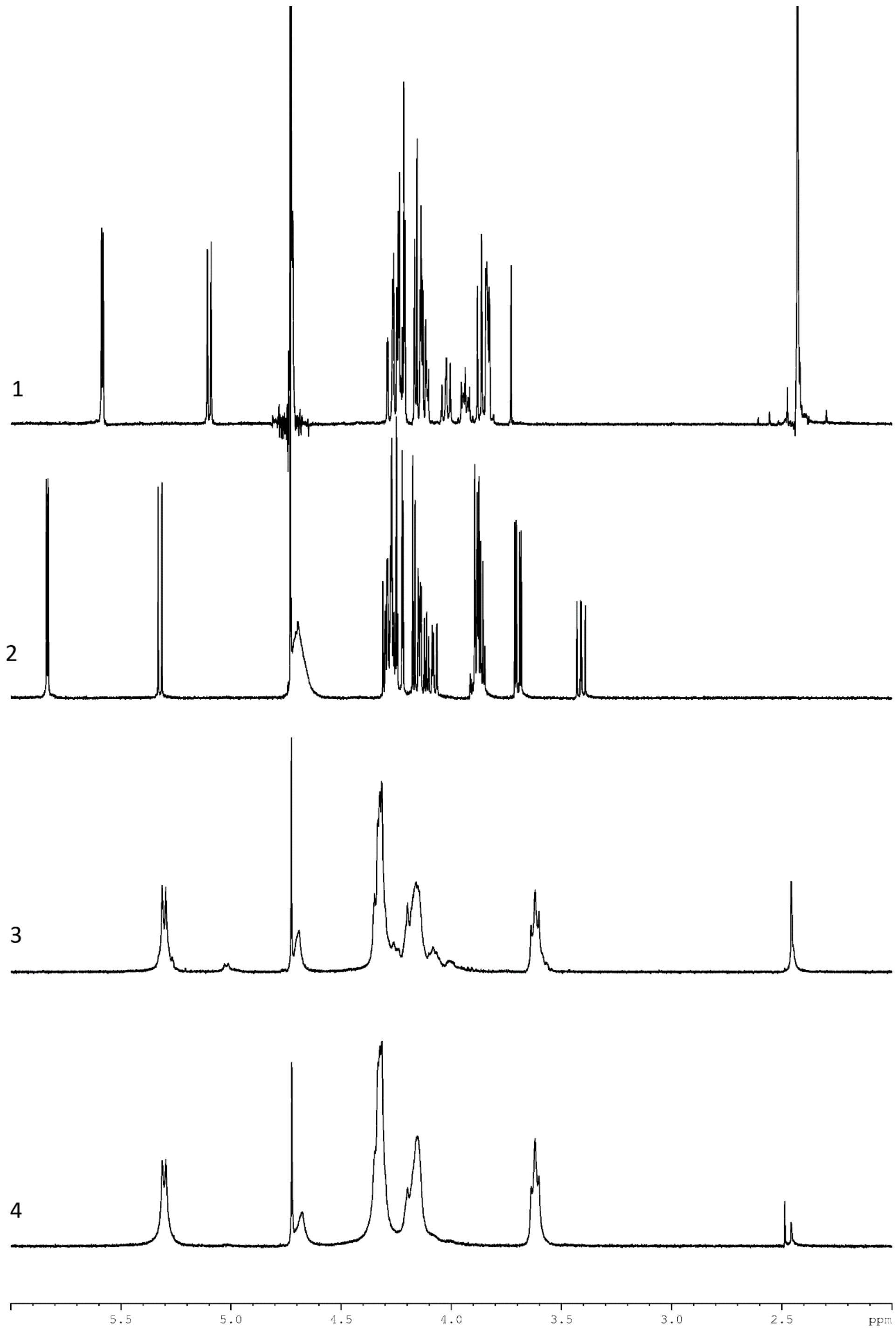


Figure 19 – ^{1}H NMR spectra for 1. *N*-acetyl-D-glucosamine (>98% assay percent range); 2. D-glucosamine (>98% percent purity), 3. 10cps ; 4. Black soldier fly derived chitosan. Deacetylation of the chitin extracted from black soldier fly was performed under homogenous conditions using 12.5 M NaOH on cycles at 60 ^{o}C for 20 hours (30 g in 1 L), repeated twice, then tested for solubility in mild acidic conditions and filtered. Chitosan was dissolved at a concentration of 6 g L^{-1} in 0.4% DCl: D_2O with TSP (3-(trimethylsilyl)propionate-d_4). TSP was used as the internal standard at a concentration of 1 mM. ^{1}H NMR was ran following Lavertu *et al.* (2003)'s experimental setup at 70 ^{o}C. 64 scans, 8 s relaxation, 90° proton pulse angle, 2 s acquisition. For the chitosan samples, H1D = integral of the peak (ppm ~5.3) corresponding to the proton attach to the carbon atom labelled in Figure 9. HAc = integral of the peak (ppm ~2.45) corresponding to the acetyl protons. H1A = integral of the peak (ppm ~4.9) corresponding to the proton attached to the carbon atom labelled in Figure 9. H26 = integral of the peaks (~3.8-4.2 ppm) corresponding to the monomer backbone protons.

Table 6 – Samples with calculated degree of acetylation using equation 1 from Lavertu *et al.* (2003). Degree of deacetylation (DDA) was calculated by equation: DDA (%) = (H-1D/ (H-1D + [H-Ac/3]))*100. (H-1D = integral 1 (ppm ~5.3); H-Ac = Integral 2 (ppm~2.45)).

Sample	**DDA (%)**	**Manufacturer DDA (%)**
10cps	87.67	94.83
Sq	93.29	93.77
NB 5cps	87.40	94.07
5cps	86.96	90.27
BSF	96.63	

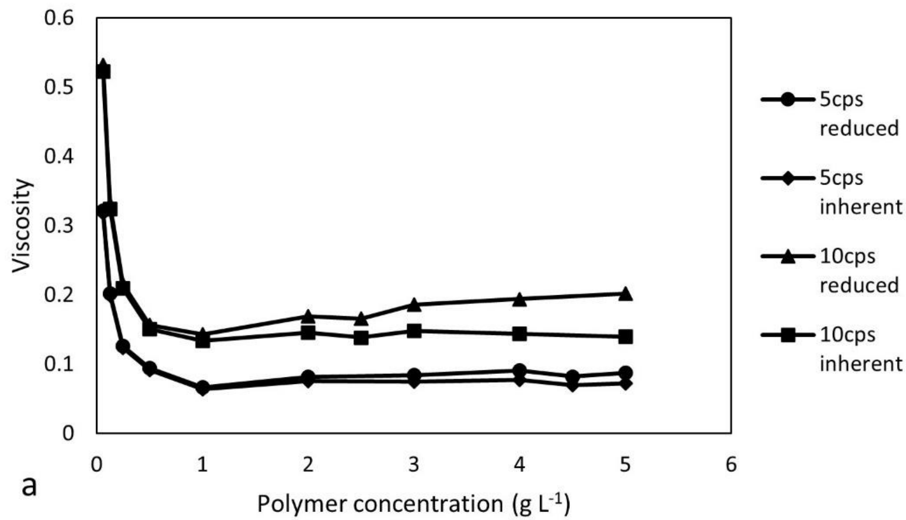
Black soldier derived chitosan had a high degree of deacetylation which measured 96.63% (Figure 19, Table 6). 5cps chitosan had a slightly lower degree of deacetylation (86.96%) compared with what the manufacturer quoted (90.27%) on the certificate of analysis. New batch 5cps chitosan also had a lower DDA (87.40%) compared to the DDA quoted by the manufacturer (94.07%). The squid derived chitosan (SqCS) measured the second highest DDA after the black soldier fly with a DDA of 93.29%. This was similar to the value the manufacturer quoted (93.77%). The 10cps chitosan measured 87.67% DDA which was

considerably lower than what the manufacturer quoted (94.83%). The spectra for the monomer units of chitosan/chitin had many peaks indicating potential issues with purity.

The commercially available chitosan's DA was measured to be different to the measurements presented on the certificate of analysis. It is unlikely that acetylation occurs over storage time, but deacetylation may be a possible degradation mechanism on top of chain hydrolysis. It may also indicate that it is difficult to achieve a consistently deacetylated sample. Therefore, separation mechanisms based on DA may be significant in refined chitosan application development in the future.

2.3.5 Mw characterisation

2.3.5.1 Initial viscometry



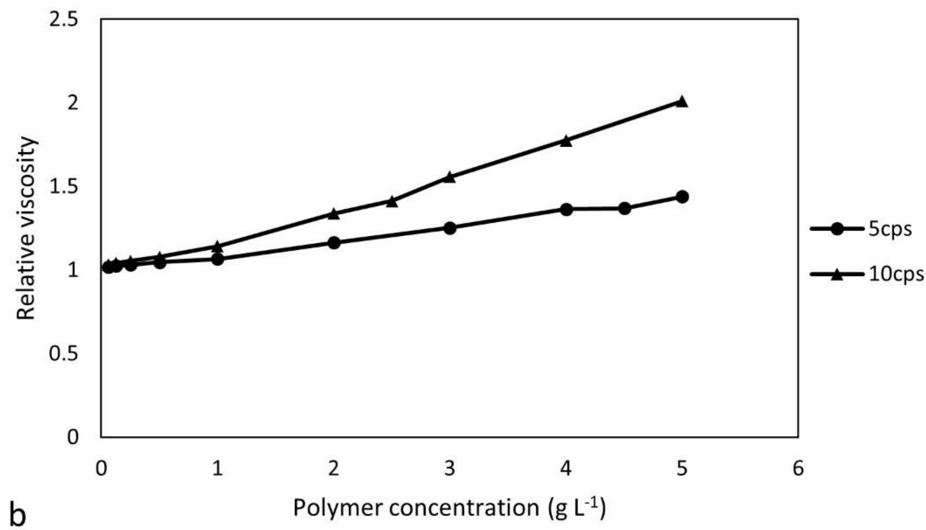


Figure 20- (a) Reduced and inherent viscosities with concentration on dilutions below the 1.2-2.5 relative viscosity exhibiting characteristic polyelectrolyte behaviour. (b) Relative viscosity plotted against concentration. As polymer concentrations approach zero, increasing dissociation and decreasing osmotic pressure cause the polymer coils to tighten. But increasing dissociation also increases coulomb repulsion forces between the charges on the polymer chain. This leads to coil expansion and the observed sharp increase in reduced viscosity (Kulicke and Clasen, 2004).

Figures 20a and 20b illustrate how polymer concentration affects the ability to measure viscosity. The angles of converging lines extrapolated from the reduced and inherent viscosity of a particular sample allow us to differentiate between valid and invalid values for

calculating the intrinsic viscosity quite simply, invalid values appear where the reduced and inherent viscosities are both increasing when extrapolating back to x = 0.

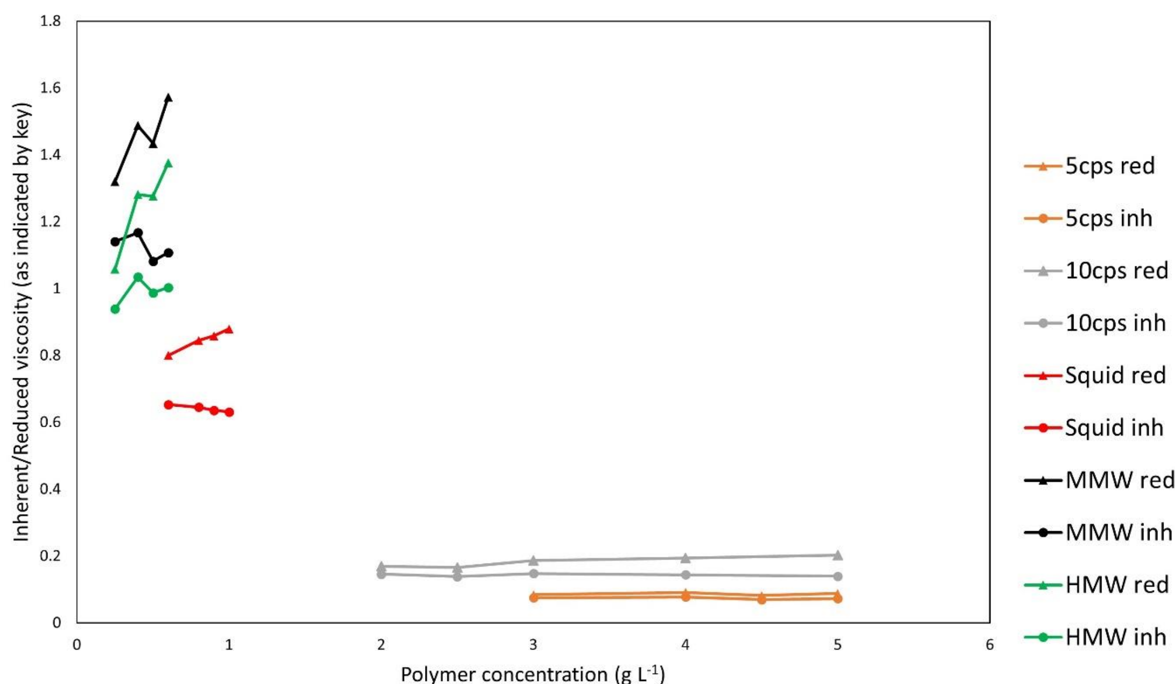


Figure 21 – Reduced and inherent viscosities for different commercial chitosan samples in 0.2 M acetic acid: 0.15 M ammonium acetate solvent (pH 4.5). Different polymer concentrations are necessary for different viscosities to achieve relative viscosities in the range of 1.2 to 2.5.

The lines are severely disjointed in Figure 21, particularly in the higher viscosity samples which makes extrapolating and inferring the intrinsic viscosity difficult. The sample with the highest centipoise value on its certificate of analysis was not measured to have the highest intrinsic viscosity. Therefore the 0.3 M HAc and 0.2 M NaAc (pH 4.5) solvent was tested following molecular weight analysis with AF4-MALS-RI.

2.3.6 AF4-MALS-RI

Three chitosan samples were successfully analysed by AF4-MALS-RI. Two commercially sourced samples followed by a BSF derived sample. The average degree of deacetylation of these samples were previously determined by ^{1}H NMR.

2.3.6.1 BSF (DDA 97%) derived chitosan.

There were two peaks present in the elution profile for the black soldier fly derived chitosan (Figures 22, 23). The aggregates comprised 1.5% of the sample. The sample without aggregates had a w-average molar mass of 1.3×10^5 g mol^{-1} ± 3.1%. The aggregates of the sample had a w-average molar mass of 2.4×10^6 g mol^{-1} ± 9.0%. Without distinguishing aggregates the w-average molar mass measured 1.6×10^5 g mol^{-1} ± 2.8%. Without distinguishing aggregates the z-average radius of gyration measured 46 nm ± 2.0%.

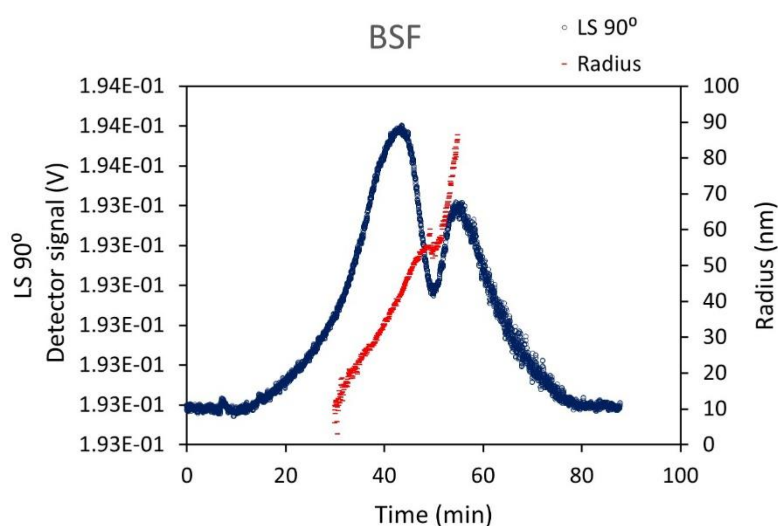


Figure 22 - Overlay plot of light scattering signal with measured radius of gyration from multi angle light scattering angular data with a random coil model for black soldier fly derived chitosan with a degree of deacetylation of 96.63%.

The molecular weight distribution of the BSF chitosan was reduced when aggregates were identified and removed from consideration. The dispersity value for the sample including the aggregates was 2.05, while the dispersity value without the aggregates was 1.69.

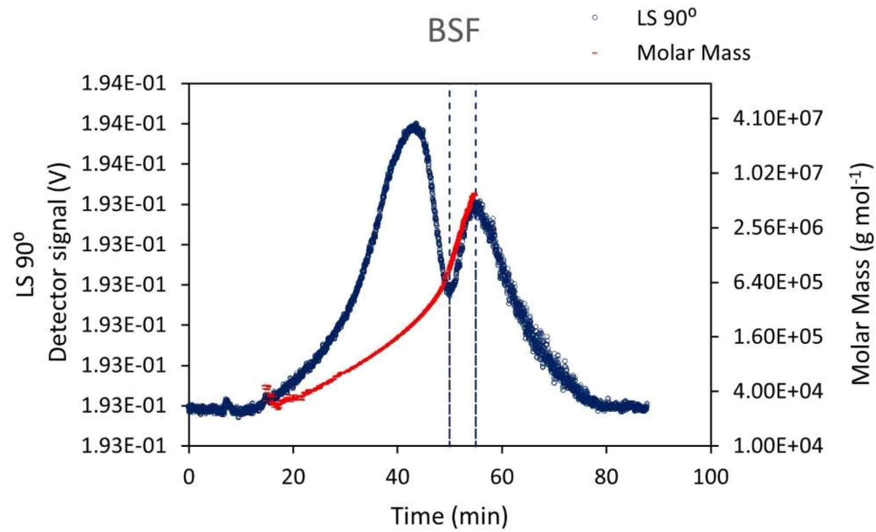


Figure 23 – Overlay plot of light scattering signal with measured molar mass from multi angle light scattering and refractive index data with a random coil model for black soldier fly derived chitosan with a degree of deacetylation of 96.63%. Aggregates are producing a signal between the dotted vertical lines.

2.3.6.2 10cps (DDA 88%) chitosan.

The 10cps chitosan sample had distinct aggregates contributing to a high radius of gyration (Figures 24, 25). The aggregates comprised 3.8% of the sample. The sample without aggregates had a w-average molar mass of 5.0 x 10^4 g mol^{-1} ± 4.5%. The aggregates of the sample had a w-average molar mass of 1.2 x 10^7 g mol^{-1} ± 13.0%. Without distinguishing aggregates the w-average molar mass measured 5.0 x 10^5 g mol^{-1} ± 4.4%. Without distinguishing aggregates the z-average radius of gyration measured 177 nm ± 1.2%.

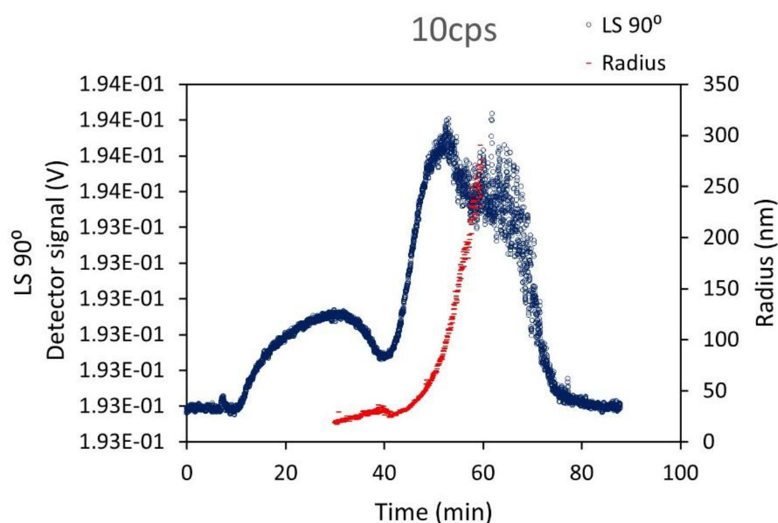


Figure 24 – Overlay plot of light scattering signal with measured radius of gyration from multi angle light scattering angular data with a random coil model for 10cps chitosan with a degree of deacetylation of 87.67%.

The molecular weight distribution of the 10cps sample was very high including aggregates. Once aggregates were identified and discounted the molecular weight distribution reduced significantly. The dispersity value including aggregates was 17.24, while the dispersity value without the aggregates was 1.79.

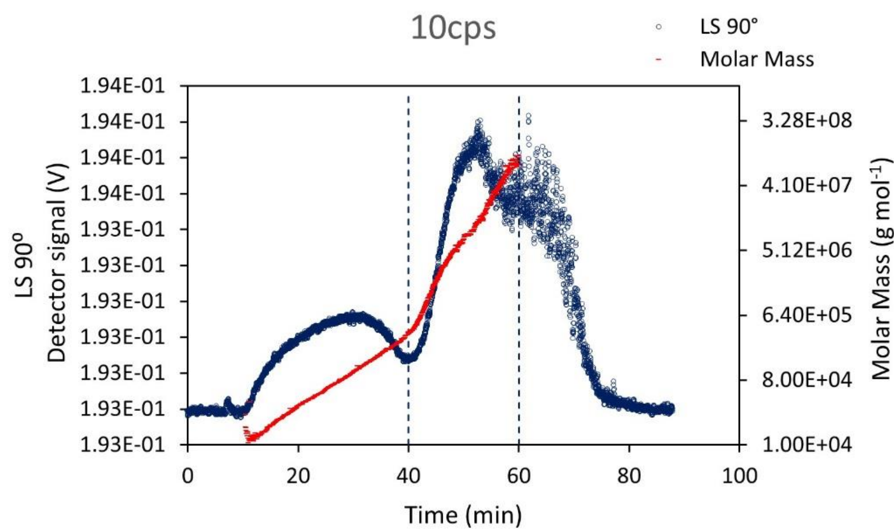


Figure 25 – Overlay plot of light scattering signal with measured molar mass from multi angle light scattering and refractive index data with a random coil model for 10cps chitosan with a degree of deacetylation of 87.67%. Aggregates are producing a signal between the dotted vertical lines.

2.3.6.3 Squid (DDA 93%) derived chitosan

The squid derived chitosan appears to have some indistinguishable peaks (Figures 26, 27). The aggregates comprised 1.0% of the sample. The sample without aggregates had a w-average molar mass of 2.2 x 10^5 g mol^{-1} ± 0.9%. The aggregates of the sample had a w-average molar mass of 6.8 x 10^6 g mol^{-1} ± 7.0%. Without distinguishing aggregates the w-average molar mass measured 2.9 x 10^5 g mol^{-1} ± <0.1%. Without distinguishing aggregates the z-average radius of gyration measured 93 nm ± 0.5%.

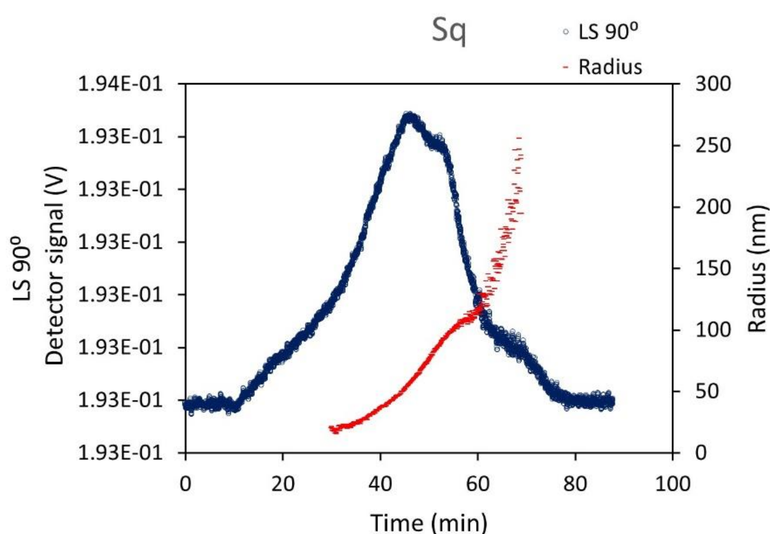
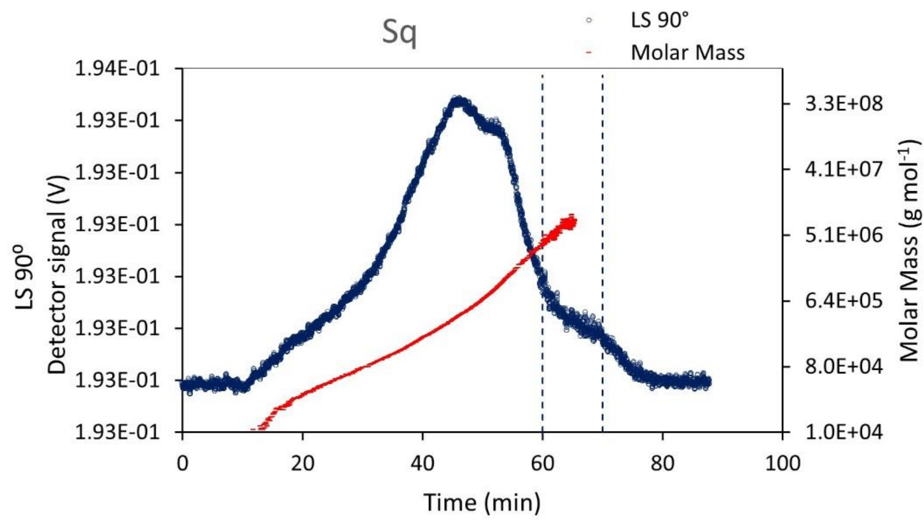


Figure 26 – Overlay plot of light scattering signal with measured radius of gyration from multi angle light scattering angular data with a random coil model for squid derived chitosan with a degree of deacetylation of 93.29%.

The squid derived chitosan had a high molecular weight distribution with and without aggregates. The dispersity value including aggregates was 5.69 while the values without aggregates was 4.31. This may be because the peaks were not as clearly defined and the aggregates were harder to identify.



Figures 27 – Overlay plot of light scattering signal with measured molar mass from multi angle light scattering and refractive index data with a random coil model for squid derived chitosan with a degree of deacetylation of 93.29%.Aggregates are producing a signal between the dotted vertical lines.

2.3.6.4 Comparing the three samples

The molar mass distribution is broad for all samples (Table 7). The BSF derived chitosan appears to have a broad molar mass distribution. This indicates that BSF could be a useful source of chitosans of different molar masses in the future alongside the development of insect bioconversion.

Table 7 – Comparison of number (n), weight (w) and centrifugation (z) average molar mass from multi angle light scattering and refractive index data for black soldier fly derived, 10cps and squid derived chitosans. Molar mass distribution is calculated by dividing the weight-average molar mass by the number-average molar mass.

Sample	**Molar mass [g mol^{-1}] (*n*-average)**	**Molar mass [g mol^{-1}] (*w*-average)**	**Molar mass [g mol^{-1}] (*z*-average)**	**Molar Mass Distribution (Mw/Mn)**
BSF	7.8 x10^{4} ± 5.3%	1.6 x10^{5} ± 2.8%	8.8 x10^{5} ± 9.8%	2.05
10cps	2.9 x10^{4} ± 4.5%	5.0 x10^{5} ± 4.4%	4.3 x10^{7} ± 5.6%	17.24
SqCS	5.1 x10^{4} ± 4.4%	2.9 x10^{5} ± <0.1%	2.6 x10^{6} ± 1.4%	5.69

The recovery was 84.1% for sample NU001, 85.9% for sample NU002 and 99.3% for sample NU003. Measurement of the samples was possible without filtration. The samples contain high molar mass material with a higher density as an indication of aggregation.

Table 8 – Comparison of number (n), weight (w) and centrifugation (z) average radius of gyration from multi angle light scattering data for black soldier fly derived, 10cps and squid derived chitosans.

Sample	**Radius [nm] (n-average)**	**Radius [nm] (w-average)**	**Radius [nm] (z-average)**
BSF	27 ± 9.1%	33 ± 5.4%	**46 ± 2.0%**
10cps	21 ± 15.0%	34 ± 6.7%	**177 ± 1.2%**
SqCS	31 ± 0.2%	47 ± <0.1%	**93 ± 0.5%**

Table 9 – Comparison of the number (n), weight (w) and centrifugation (z) average molar mass of polymer and aggregate fractions for black soldier fly derived, 10cps and squid derived chitosan samples. Molecular weight distribution was calculated by dividing the weight average molar mass by the number average molar mass.

Sample	Fraction	Molar mass [g mol^{-1}] (*n*-average)	Molar mass [g mol^{-1}] (*w*-average)	Molar mass [g mol^{-1}] (*z*-average)	Content based on Area of RI Detector (%)	Molar Mass Distribution (Mw/Mn)
BSF	1st Fraction (polymer)	7.7 x10^{4}^ ± 5.1%	**1.3 x10^{5} ± 3.1%**	2.2 x10^{5} ± 1.4%	98.50	1.69
	2nd Fraction (aggregates)	1.8 x10^{6}^ ± 5.7%	**2.4 x10^{6} ± 9.0%**	3.2 x10^{6} ± 12.0%	1.50	
10cps	1st Fraction (polymer)	2.8 x10^{4} ± 7.2%	**5.0 x10^{4} ± 4.5%**	8.7 x10^{4} ± 7.5%	96.20	1.79
	2nd Fraction (aggregates)	1.1 x10^{6} ± 18.0%	**1.2 x10^{7} ± 13.0%**	4.7 x10^{4} ± 4.2%	3.80	
SqCS	1st Fraction (polymer)	5.1 x10^{4} ± 5.1%	**2.2 x10^{5} ± 0.9%**	9.2 x10^{5} ± 2.2%	99.00	4.31
	2nd Fraction (aggregates)	5.8 x10^{6} ± 12.0%	**6.8 x10^{6} ± 7.0%**	8.2 x10^{5} ± 0.3%	1.00	

Table 10- Comparison of structure from conformation plots for black soldier fly derived, 10cps and squid derived chitosans. MW- molecular weight.

Sample	Overall slope	Low MW region	High MW region
BSF	0.339	0.719	0.176
10cps	0.386	0.575	0.490
SqCS	0.465	0.685	0.365

The aggregates can be identified by the second peak in the elution profiles (Figures 28, 29), contributing to a higher average molar mass and radius of gyration (Tables 8, 9). The conformation plots highlighted the presence of aggregates in the sample due to a low gradient for the line of best fit (α value) (Table 10).

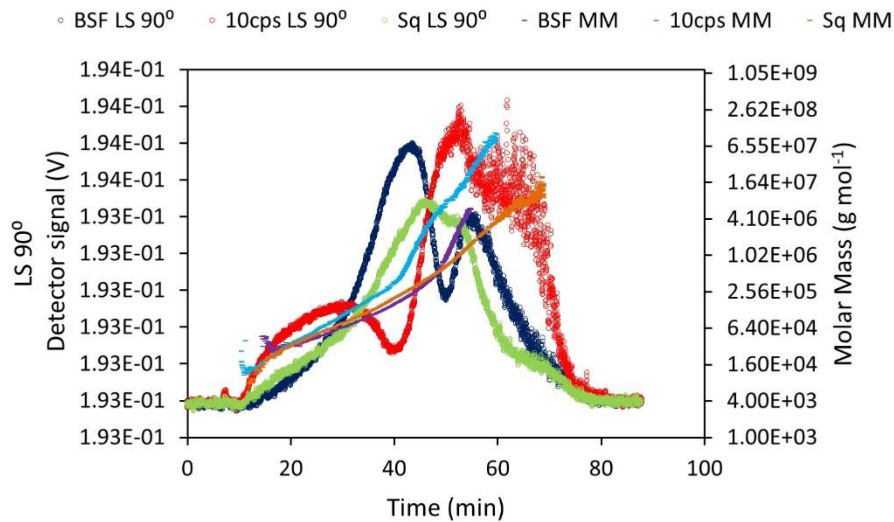


Figure 28 – Comparative plot of the light scattering signal and molar mass (g mol^{-1}) of black soldier fly derived, 10cps and squid derived chitosan samples.

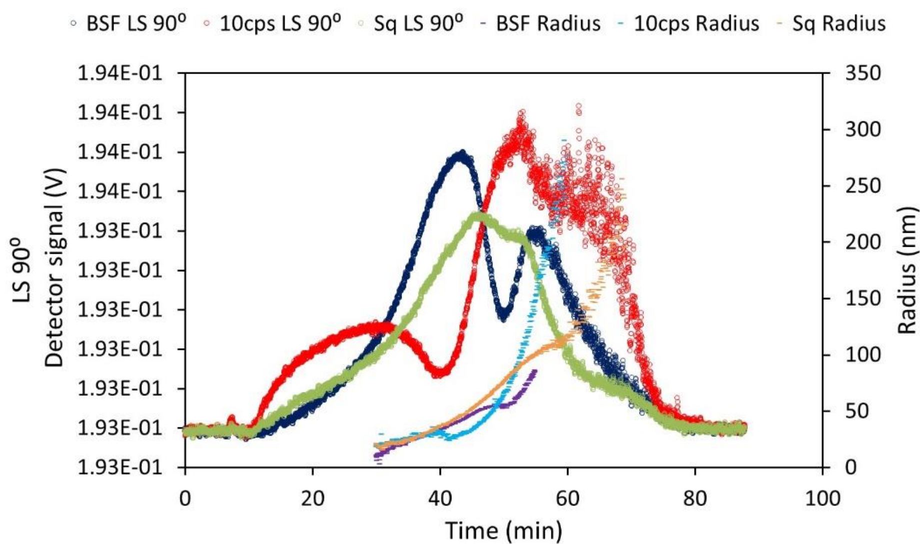


Figure 29 - Comparative plot of the light scattering signal and radius of gyration (nm) of black soldier fly derived, 10cps and squid derived chitosan samples.

2.3.7 Viscometry

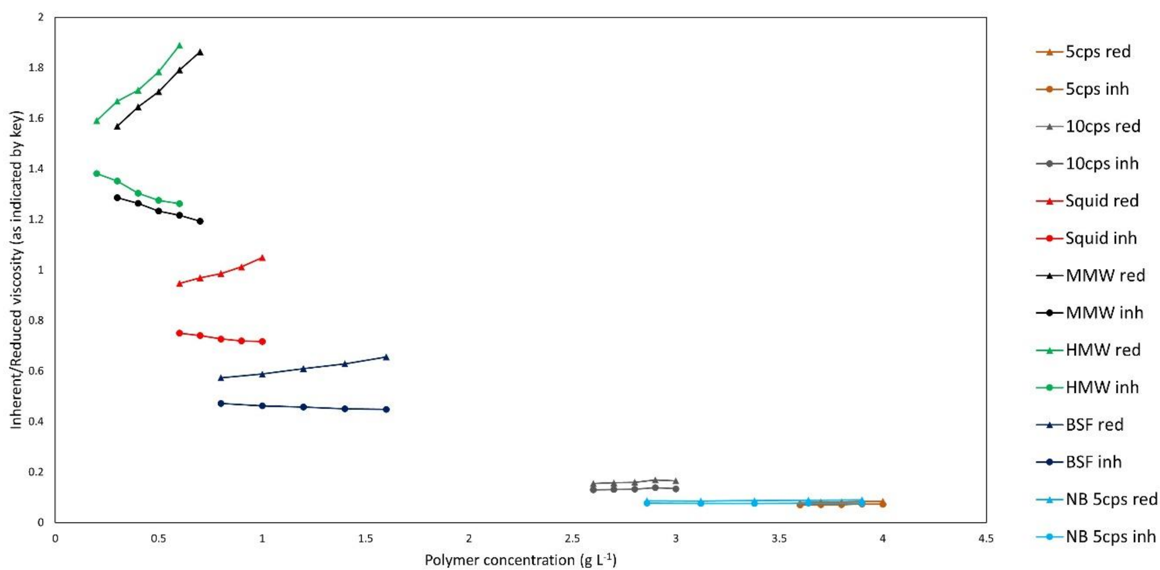


Figure 30 – Inherent and reduced viscosities of various chitosan samples dissolved in sodium acetate buffer (0.3 M HAc: 0.2 M NaAc pH 4.5) to permit extrapolation for the intrinsic viscosities.

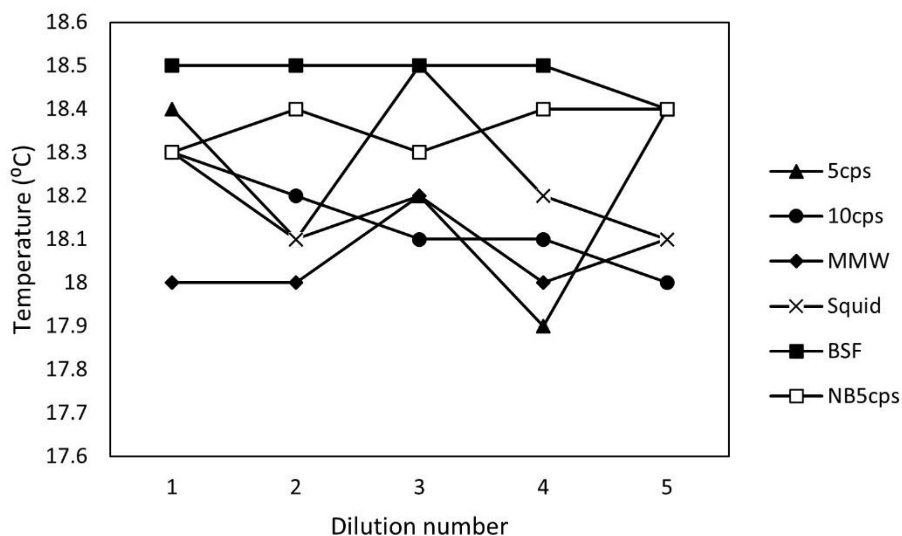


Figure 31 – Variability of temperature throughout the dilution measurements of chitosan samples during dilute solution viscometry using an Ubbelohde viscometer with a 0.58 mm capillary diameter. Solvent used was 0.3 M HAc: 0.2 M NaAc pH 4.5.

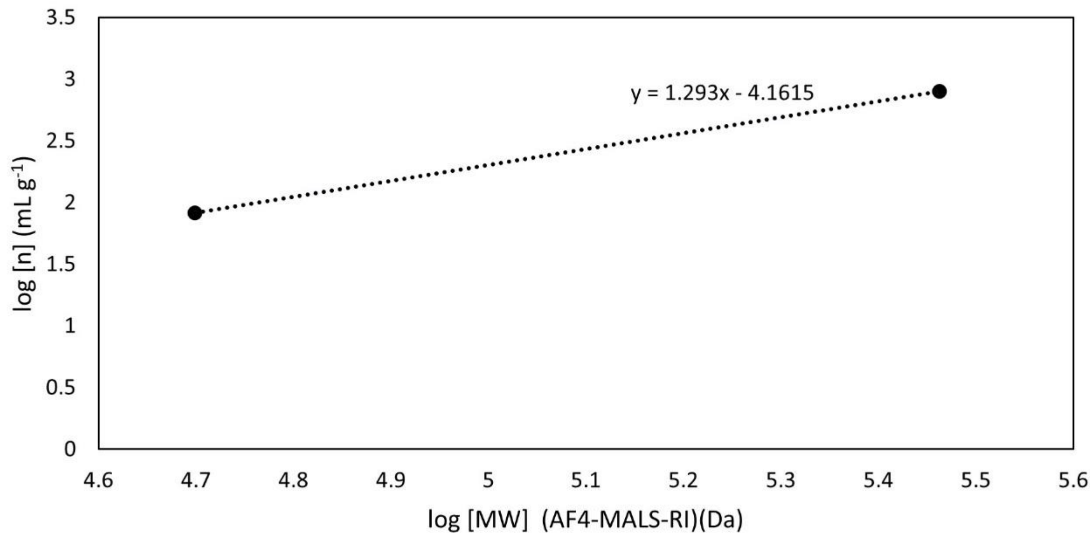


Figure 32 – Logarithm of the intrinsic viscosities plotted against the logarithm of the molar masses from AF4-MALS-RI enables deduction of the system specific Mark-Houwink constants K and α for the specific system setup.

Intrinsic viscosity values for chitosan samples measured through capillary viscometry (Figures 30, 31) fit the same pattern as the centipoise values on the certificates of analyses. Estimated molar masses from centipoise values are quite similar for 10cps chitosan but not similar for squid chitosan. Figure 30 shows the reduced and inherent viscosities for chitosan samples while Figure 31 shows the temperature variability during the measurements.

From the Mark-Houwink constants derived using the 10cps and Squid chitosan values from AF4-MALS-RI and viscometry (Figure 32), an estimation from the intrinsic viscosity of the BSF derived chitosan could be made. This resulted in an estimated molar mass of 199240 Daltons which is relatively comparable to the average molar mass directly measured through MALS (130-160 kDa) [490.45 = [n]][α = 1.293; K=0.0000689 (mL g^{-1}); [n] /K = M^{α}](MW = 199240.3128). However this estimation is not as considerably different when comparing the estimation provided for squid chitosan from a centipoise value (estimated at 580 kDa, measured MALS average = 290 kDa).

2.4 Discussion

2.4.1 Extraction

2.4.1.1 DES vs Conventional extraction

From the results obtained the conventional extraction yield was accurate compared with previous studies. Zhou *et al.* (2019) used the conventional extraction method to determine chitin content of BSFP and reported a yield of 6.5%. As an alternative method, Caligiani *et al.* (2018) adapted a procedure from Flannery *et al.* (2001) to quantify the chitin present in the BSFP sample. To avoid the loss of water soluble chitin components, they hydrolysed the entire sample and quantified the glucosamine residues through GC-MS. This provided a value of 9% chitin from BSFP powder. The conventional extraction yield (10.68%) is most similar to this and also falls within a similar range to that achieved by Hahn *et al.* (2018) (5-15%), however it is higher than Spranghers *et al.* (2017) (5.5-7%), Smets *et al.* (2020) (3-6%) and Zhou *et al.* (2019) (6.5%) yields.

In addition, there is a higher yield of chitin from the DES extraction compared to conventional extraction and those reported by other researchers (Caligiani *et al.*, 2018; Zhou *et al.*, 2019). Zhou *et al.* (2019) reported a yield of 22.8% when they performed the choline chloride/urea DES extraction and measured a purity of 86.5%. This is considerably different to our results which indicates that the extraction may be more efficient at separating chitin material. However, it could also mean that the reaction has not performed well and there may be a greater quantity of contamination. Therefore, further analysis is necessary.

2.4.1.2 Conventional extraction

Compared with overall percentage mass yields from previous studies of chitin extraction from larval stages of the BSF (Hahn *et al.*, 2018; Smets *et al.*, 2020; Spranghers *et al.*, 2017; Zhou *et al.*, 2019) (5-15%, 3-6%, 5.5-7%, 6.5% respectively), this study falls into a similar range. Caligiani *et al.* (2018) (9%) is the main overestimated value for yield however their method was not an extraction but was based on a hydrolysis step of raw BSF material and quantification of a glucosamine peak in GC-MS. This indicates that only one step was used and there was a lack of wash and transfer steps.

The chemical steps for extraction are likely to have an impact on the chain and may cause some degree of hydrolysis which may increase sample loss due to water soluble fragments. While chitin is known to be relatively tough, it is still a biopolymer. Alginate for example can

be hydrolysed easily for ^{1}H NMR analysis in extremely mild acid treatments (Grasdalen, 1983).

Soluble chitosan was successfully produced from whole black soldier fly prepupae using a conventional chemical extraction method followed by homogenous deacetylation conditions with a soluble chitosan yield of 28.31%. Other studies that have extracted chitosan from insects in general are limited. One previous paper has produced chitosan from black soldier fly derived chitin (Hahn *et al.*, 2020), which compared different reaction conditions and followed the homogenous reaction based on Nemtsev *et al.* (2002). Previous papers that have produced chitosan from other insects have used similar conditions with strong NaOH at 70-140 ^{o}C (Ai *et al.*, 2008; Jing *et al.*, 2007; Nemtsev *et al.*, 2004).

Interestingly, Hahn *et al.* (2020) compared a number of deacetylation conditions and reported chitosan yields and properties accordingly. Though still to date, no previous literature has examined insect derived chitosan molar mass distribution or directly measured the molar mass through light scattering. Three 'heterogenous' and one 'homogenous' deacetylation reactions were compared by Hahn *et al.* (2020) using 12 M NaOH and 10 M NaOH respectively. Either route involves extremely harsh chemical conditions which may result in severe chain degradation and therefore subsequent loss of sample and poor chitosan yields.

Hahn *et al.* (2020) heterogenous methods resulted in chitosan yields of 22, 38, and 47% and the homogenous reaction resulted in 13%. This study resulted in a chitosan yield of 28.4% using homogenous conditions, which is most comparable to the heterogenous yields. In fact there is a wide range of yields when comparing with Hahn *et al.* (2020) for various deacetylation reactions. Future studies could benefit from thorough optimization of this yield for scaling up production.

2.4.2 ^{1}H NMR for determination of binary heteropolysaccharide composition

2.4.2.1 M/G ratio of food grade alginate

The food grade alginate was determined to have a guluronic acid (G) monomer content of 30.9% and a mannuronic acid (M) content of 69.1%. Similarly to chitosans degree of acetylation the composition of the copolymers of alginate have implications for its functionality. For example differing copolymer compositions may impact the affinity to form

polyelectrolyte complexes with chitosan (Conzatti *et al.*, 2017). Copolymer composition dependent effects have been evidenced for alginate in its applications (Lee and Mooney, 2012). The G content is known to have a relationship with an increase in the inter molecule cross-linking of the polymer (Lee and Mooney, 2012) and will therefore impact its conformation in solution, and its viscosity. This sample appears to have a relatively low G content and a high frequency of consecutive mannuronic acid residues (Fmm = 47.78%) which may have implications for its molecular interactions.

2.4.2.2 DA of chitosan

Black soldier fly derived chitosan was the most deacetylated chitosan measured. In this study, the BSF derived chitosan had a DDA of 96.63% which is significantly higher than the previously produced BSF derived chitosan by Hahn *et al.* (2020). This could be due to the repetition of the alkali treatment three times. It could also be due to the duration of the reaction. The reaction was also homogenous but not diluted with ice like Nemtsev *et al.* (2002) and Hahn *et al.* (2020). Therefore, there are quite significant differences in conditions for homogenous reactions.

Hahn *et al.* (2020)'s samples had measured degrees of deacetylation of 34, 44 and 72% for the heterogenous, and 34% for the homogenous. These were measured by titrations and are interesting as the solubility of a heavily acetylated chitosan/chitin is usually more soluble at neutral or even alkali pH (Rinaudo, 2006) unless they are of low molecular weights. However, these appear to be soluble in 1% acetic acid and measured through viscometry also. This may raise questions about the titration method for DA determination.

In addition to this, titration is a popular method with several other insect chitosan papers. Ai *et al.* (2008) measured a DDA of 90.3% with titration for chitosan extracted from *Musca domestica.* This study deacetylated chitin in similar conditions to our study which highlights that it is effective for producing highly deacetylated chitosan. Unfortunately Ai *et al.* (2008) did not report their yield of chitosan from chitin so the efficiencies of the reactions cannot be compared.

Finally, the names of reactions lead to false interpretations of the measured degree of acetylation/deacetylation of the samples. There is a distinct lack of evidence that the chitosan samples produced by the different reactions actually produce homogenous or

heterogeneously acetylated chitosans. It needs to be made clear that these reaction titles refer only to the conditions that the chitin is exposed to, and does not refer to the deacetylation pattern of the final product. An interesting method using capillary electrophoresis could be pivotal in further analysing the differences within samples from the same reaction (Thevarajah *et al.*, 2017).

2.4.3 Molecular weight

2.4.3.1 Viscometry

Constants for the Mark-Houwink equation were calculated using the logarithms of the molar masses and intrinsic viscosities. The Mark-Houwink 'α' constant is quite high, suggesting a rigid chain characteristic. This contrasts to previous studies with lower values.

The constants of the Mark-Houwink equation require experimental determination. The α constant is a measure of solvent quality and K is dependent on α (Kulicke and Clasen, 2004). Our value for α (1.293) was slightly higher than previously reported α values (see Table 1). It was most similar to Roberts and Domszy (1982) with their α=1.26 for the solvent 0.1 M HAc with 0.02 M NaCl at 25 ^{o}C. Their corresponding K value was 0.0000304 which was also of a comparable magnitude to our K value (0.0000689). When comparing the studies using the same solvent we used, González-Espinosa *et al.* (2019) had the most similar K (0.0000229) and α (1) values for their chitosans with lower molecular weights and lower degrees of acetylation at 35 ^{o}C.
Previous studies have most commonly been performed at higher temperatures such as 25 ^{o}C. The Mark-Houwink 'K' value is very low but comparable to some previous literature. Temperature may be impacting the polymer conformation which may have an influence on the constants. Chitosan chain stiffness has been shown to decrease when the temperature increases (Rinaudo, 2006). Therefore the high α constant could be explained by the inverse of this behaviour: stiffness increase with lower temperatures.

Furthermore, the samples may not be consistently deacetylated and as the measurements are an average, there is going to be a level of inter and intra sample variability. The DA variation has been studied for its impacts on the properties of the polymer in solution and there is no known influence of DA on the chain properties, including the rigidity (Rinaudo, 2006). However, the ionic charges increase with a decreasing DA, which may have an

influence on the aggregation of the polymer as well the molecular weight as earlier discussed (Rinaudo, 2006).

2.4.3.2 AF4-MALS-RI

The squid derived chitosan appears to have some indistinguishable peaks. This could be aggregates present. The pH 3.7 acetate buffer used by González-Espinosa *et al.* (2019) may have gave better definition to the peaks.

There seems to be differences in the degree to which the samples aggregate, suggesting that certain polymer characteristics may encourage aggregations more so than others. This may be in agreement with some other experiments (Kang *et al.*, 2021; Yanagisawa *et al.*, 2006). For example the aggregations appear more clearly in the lower molar mass samples. The aggregates also appear most distinct in the sample with the highest degree of acetylation. Whereas the aggregates are harder to distinguish from the main sample in the highest molar mass sample. However, the quality of the solvent has been the topic of debate regarding aggregates for a considerable amount of time also (González-Espinosa *et al.*, 2019; Philippova *et al.*, 2001; Philippova and Korchagina, 2012; Rinaudo, Milas and Le Dung, 1993).

Comparing the percentage of the sample that the aggregates are taking up, means they would not contribute significantly to viscometric studies. Applying vertical lines to separate the peaks allows average molar masses to be calculated without including aggregates. These molar mass values can be used to assess the constants for the Mark-Houwink equation in combination with viscometry data. Chitosan aggregates have long been a subject of discussion in research literature. Some solvents are more effective at reducing aggregation than others.

2.4.3.3 Addressing aggregations

AF4 has received previous attention in chitosan research for its ability to identify and allow quantification of chitosan aggregations (González-Espinosa *et al.*, 2019). Theories around chitosan’s tendency to aggregate have been hypothesised for several years and is still not fully understood.

One of the crucial factors that may suggest viscometry to be a more reliable method is the fact that the dilution regime is not being modified. As opposed to AF4 and SEC systems the aggregation could be caused by ultra-dilution due to additional solvent to an already dilute

polymer solution. This may cause chain expansion for smaller molecules more readily than larger molecules as they may leave their Newtonian plateau at a higher concentration than the higher molar mass samples. Therefore increasing persistence length in the lower molar mass samples would increase its likelihood of collision with other chains and in turn increase the likelihood of aggregations occurring between chains.

An example of this is Figure 20a which highlights the end of the Newtonian plateau for SqCS at a concentration less than 2 g L^{-1} which is the starting concentration for the AF4 analysis. Whereas the BSF and Squid chitosans were both measured below 2 g L^{-1} in Figure 30.

To further investigate this, a comparison study using a capillary viscometry setup in a more refined temperature controlled bath, exploring the range of concentrations that a chitosan sample is likely to encounter in one round of AF4 would be useful. Then a parallel study using AF4 (same buffer, pH and starting concentration) to quantify aggregations at different polymer concentrations inside and exiting the Newtonian plateau for that specific sample.

2.4.4 Conclusions and future directions

2.4.4.1 Extractions

Achieving full solubility in the DES experiment proved difficult. This may be due to the life stage being used. The exoskeleton of the prepupae was dark and hardened which may indicate more advanced signs of melanisation and sclerotization. Selecting the life stage before this change could help in understanding whether there is an optimal life stage that pure BSF chitin can be extracted. If the life stage is not providing a desirable quantity of protein and fats, the chitin may require more thorough processing to remove more persistent contaminants.

In addition, previous studies have used different compositions of DES that have had different effects on the removal of minerals and proteins with different arthropods. Exploring more DES combinations using BSFP may give a better purity that could be useful in the future.

There is a need for further method optimisation for BSF specific substrates. And a need to understand the stability of chitin in the extraction conditions to allow a more reliable quantification of yield.

2.4.4.3 Molecular weight analysis

As chitosan research advances, there is a need for defined chitosans with refined molecular weights. There is therefore also a need for a detailed study regarding the stability of chitosan in solution and estimations for the time it may take to degrade in the natural environment. This could be facilitated by monitoring the molecular weight distribution of a sample over a significant period of time in varying conditions. This would be beneficial in the developmental process for companies interested in producing chitosans for specific applications.

Chitosan from the BSF has not previously been studied for its molecular weight distribution. But shows similarities to other sources and also is evidently prone to some aggregations. The molecular weight of chitosan derived from insects has been measured by other indirect methods.

There are still clearly aggregations occurring when trying to analyse chitosans by AF4. Over many years of research there have been proposed conditions which minimise these aggregations but it is now clear that they were not prevented but rather went undetected. There is therefore a need for a better understanding of how aggregations are occurring in different solutions.

2.5 References

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Chapter 3. Antimicrobial activity assessment

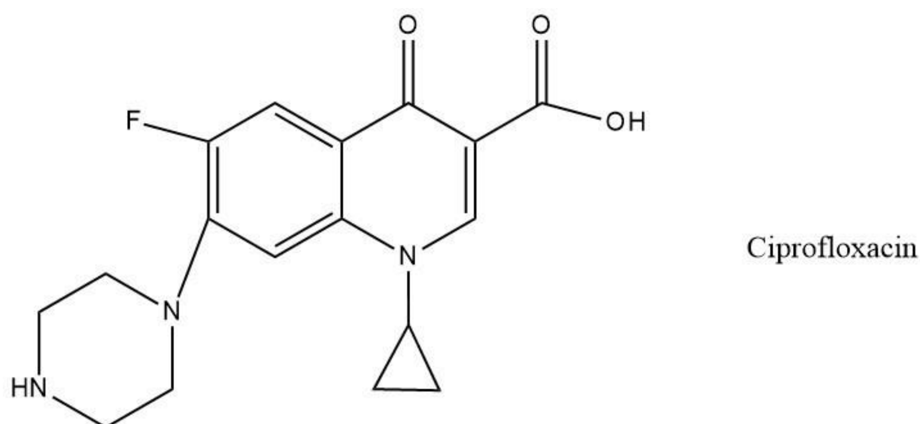
3.1 Introduction

3.1.1 Antimicrobials

Antimicrobials have uses in many areas including medicinal and food applications. Penicillin was one of the first antimicrobials discovered for medicinal purposes in 1928 by Alexander Fleming (Fleming, 1929). As society has advanced, studying antimicrobial activity has allowed the production of further potent antimicrobial molecules but has also identified risks of antimicrobial resistance. Antimicrobial resistance has become an issue due to the misuse of antimicrobials (Reygaert, 2018).

Characterising the antimicrobial activity of a molecule can help provide direction for its application. This can be highlighted by understanding the mode of action, the species which are sensitive to the molecule and the risks associated with using the molecule such as the likelihood of inducing antimicrobial resistance mechanisms, and whether the molecule can be optimised or altered to improve its efficacy.

Different mechanisms of antimicrobial activity have allowed an array of antimicrobial molecules to become available on the market with different efficacies against different organisms. Different antimicrobial mechanisms include those that can disrupt the cell surface membrane such as lipopeptides (Reygaert, 2018). Some molecules, such as quinolones, can interfere with the DNA of cells and some can inhibit protein synthesis, for example the aminoglycosides (Reygaert, 2018) (Figure 33).



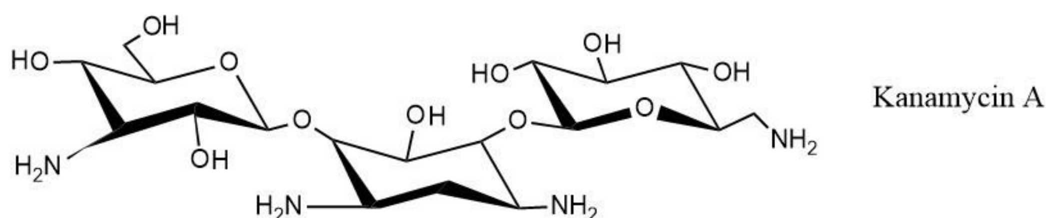


Figure 33 – Examples of the structures of a fluoroquinolone (Ciprofloxacin) and an aminoglycoside (Kanamycin A).

There are structure-activity relationships between an antimicrobial molecule and the mechanism of action it imposes on bacterial cells (Czaplewski *et al.*, 2009; Haydon *et al.*, 2008). Understanding the activity can therefore help in understanding how different alterations to the structure of an antimicrobial can improve or hinder its activity. One important consideration is how a molecule interacts with and bypasses the cell wall.

3.1.2 Bacterial cell walls

Bacterial cell walls are an extremely complex feature that provide structural integrity, a protective barrier to the cytoplasmic membrane and shape to individual cells (Dörr, Moynihan and Mayer, 2019). Bacterial cell walls consist of peptidoglycan (PG), which consists of crosslinking peptides and polysaccharides, providing structural rigidity and the ability to withstand turgor (Vollmer, Blanot and de Pedro, 2008).The polysaccharides present in PG are mainly alternating *N*-acetyl-ᴅ-glucosamine and *N*-acetylmuramic acid sugars (Figure 34)(Vollmer, Blanot and de Pedro, 2008).

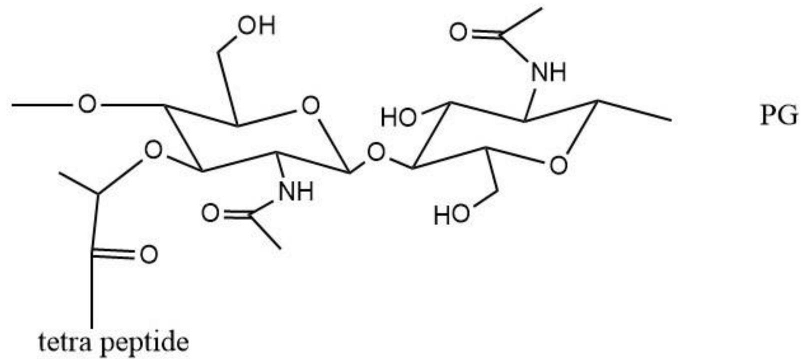


Figure 34 – The structure of the alternating sugars *N*-acetyl-ᴅ-glucosamine and *N*-acetylmuramic acid present as the backbone of peptidoglycan.

Both gram positive and gram negative bacteria possess cell walls, although they have some distinct differences which permit them to be differentiated by Gram staining with crystal violet where the thick cell wall of gram positive bacteria retain the stain and gram negative bacteria fail to retain it (Silhavy, Kahne and Walker, 2010). Gram positive bacteria have thick cell walls made up of PG and teichoic acid chains extending through the wall from the surface of the plasma membrane that play a role in cell division (Swoboda *et al.*, 2010). Gram negative bacteria have a much thinner PG layer but they also have an outer membrane consisting of lipopolysaccharides (LPS) which provide further protection (Silhavy, Kahne and Walker, 2010). LPS are made up of a polysaccharide and an 'O' antigen which help tolerate environmental stressors (Raetz and Whitfield, 2002). The outer membrane and cell wall are linked to one another through murein lipoproteins (Braun, 1975).

It is clear that damaging the bacterial cell wall integrity would result in cell death (Egan, Errington and Vollmer, 2020). Therefore molecules with novel mechanisms that disrupt its structure and metabolism are a prize target.

3.1.3 Food associated bacteria

There are numerous bacteria which are associated with the degradation or contamination of substances. Bacteria present in foods can lead to shelf life limiting degradation or health issues for consumers.

Injurious pathogens are can become present on foods through poor hygiene practices during preparation (EC, 2005). For example *Escherichia coli* has strains which are a public health concern such as verotoxigenic *Escherichia coli* (VTEC) (EC, 2005). *Listeria monocytogenes* can

cause severe health problems for immunocompromised individuals (FSA, 2022). *Campylobacter* and *Salmonella* are associated with certain food types and can cause food poisoning symptoms (FSA, 2018a; FSA, 2018b; Marder *et al.*, 2017).

Food degradation and consumer concern about the freshness of food are significant contributors to food waste (Neff, Spiker and Truant, 2015; Parry, Bleazard and Okawa, 2015). Degradation of food can lead to visual differences such as colour changes (FSA, 2010). Injurious pathogens are not easily detected and require microbiological and molecular biological testing to confirm their presence as part of investigations and precautionary measures (EC, 2005; Marder *et al.*, 2017).

It is useful to compare gram positive and gram negative bacteria against chitosan activity to gain a better understanding of the types of bacteria that may be susceptible to chitosans. Common food associated bacteria include gram negatives such as *Salmonella* and *Pseudomonas*, and gram positive bacteria such as *Bacillus* and *Listeria* (EC, 2005; FSA, 2010). It is important to cover a range of taxa as it increases metabolic diversity and potential weaknesses in antimicrobial activity and therefore invalid applications.

3.1.4 Antimicrobial polymers

Antimicrobial polymers can be divided into natural and synthetic. Chitosan is a cationically charged biopolymer which means it may have similar activity to other cationically charged polymers such as polylysine and polyethylene imine (PEI).

PEI has been developed as a synthetic cationic pharmaceutical excipient. Gibney *et al.* (2012) examined the antimicrobial activity of differently branched and polymerised PEI against *Staphylococcus aureus* and *E. coli*. In their membrane permeabilization assays they concluded that the membrane disruption was not a component of the mode of action. They also noted that PEIs were selective to bacteria over red blood cells and in addition to this they found that there was enhanced growth inhibition for PEIs against *S. aureus* when compared with *E. coli*. These conclusions highlight that the mode of action is targeting bacterial cells specifically and more so targeting processes associated with gram positive bacteria over gram negative bacteria. Further to this, Mikula *et al.* (2018) performed comparisons of the selectivity of branched PEI’s to algal and cyanobacterial cells. Their results highlighted that the branched PEIs have taxa specific impacts as they found certain

species flocculated more upon treatment than others. This is significant because it shows a structure-activity relationship for cationic polyelectrolytes towards certain taxa which could be further developed for biotechnological applications.

Polylysine has applications in foods and is considered safe for food applications in several countries (Lopez-Pena and McClements, 2014). The antimicrobial impacts of this polymer have also been studied. El-Sersy *et al.* (2012) compared the inhibitory effects of polylysine against several gram positive and negative bacteria (*S. aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and several *Vibrio* sp). They found that polylysine inhibited all species except *S. aureus*. Another interesting study on polylysine by Lopez-Pena and McClements (2014) highlighted the limitations of polycations as antimicrobial due to their interactions with anions. This is particularly relevant to food contact applications due to the use of several anionic polysaccharides in foods as stabilisers. When comparing polylysine complex formation between carrageenan and pectin, they found differences in complex displacement. This is significant because certain displacement preferences may indicate enhanced binding affinity between two molecules which may result in different properties.

3.1.5 Antimicrobial activity methods and reporter strains

There are two main routes for discovery of antimicrobial compounds; target based and whole cell based screening. Target based screening involves identifying a molecule which inhibits a specific reaction that an organism relies on for function, but does not necessarily identify molecules that can work as a drug (Rosamond and Allsop, 2000). Whole cell screening uses live bacterial cells to indicate whether a compound is inhibitory or not to that specific strain. This route recognises functional antimicrobials that work effectively but requires identification of the mode of action before it can be optimised (Rosamond and Allsop, 2000).

Antimicrobial activity of a molecule can be measured in culture dependent susceptibility methods in whole cell screening with agar and broth based methods. Broth microdilution methods are typically the most accurate method for deducing antimicrobial activity as they can allow the calculation of minimum inhibitory concentrations (MICs) of a specific molecule (Balouiri, Sadiki and Ibnsouda, 2016). This is defined as the smallest concentration of an antimicrobial that prevents visible growth of a specific microorganism following overnight

incubation (Andrews, 2001). Given that chitosan samples are a mixture of molecules, determining an MIC may be not be exact due to the unknown proportions present. Disc and agar well diffusion methods are suited for extracts (Boyanova *et al.*, 2005; Valgas *et al.*, 2007) and chitosan (Jing *et al.*, 2007; Younes *et al.*, 2014) as they can be performed as bioassays on larger agar plates, meaning that they can be high throughput, which is particularly useful when optimising sample composition. Additionally it has been noted that agar well diffusion is more sensitive than disc diffusion for certain antimicrobials , most likely due to the possible increase in sample load (Boyanova *et al.*, 2005; Valgas *et al.*, 2007).

Whole cell screening based analysis has been developed further to produce live cell biosensors that can indicate which antimicrobial mechanism is stressing them (Hutter *et al.*, 2004; Urban *et al.*, 2007). They have high throughput and can be performed easily with low compound concentrations (Hutter *et al.*, 2004). This permits a general antimicrobial mechanism to be highlighted for further focussed study.

3.1.6 Reporter strains

Bacterial reporter strains have been developed to aid in the diagnosis of antimicrobial mechanisms of action for antimicrobial development (Hutter *et al.*, 2004; Urban *et al.*, 2007). Reporter strains are designed to highlight a positive result to a certain mechanism of action. Testing against a variety of strains therefore screens the molecule to various actions (Hutter *et al.*, 2004). Reporter strains are living cells that have been genetically modified. The promoter for a gene of interest is attached to a reporter gene and can be used to detect certain responses (Hutter *et al.*, 2004; Urban *et al.*, 2007).

When promoters for genes of interest associated with responses to certain antimicrobial activity are used with a reporter gene, the reporter protein is produced in response to the gene of interest promoter becoming active (Hutter *et al.*, 2004; Urban *et al.*, 2007). This reporter protein is usually measurable or detectable.

Various reporter strains have been reported in the literature using different reporter genes. In particular, *Bacillus subtilis* 168 has received a great deal of attention due to it being a non-pathogenic strain. Ulijasz, Grenader and Weisblum (1996) used *B. subtilis* 168 with a LacZ reporter. Fischer *et al.* (2004) examined *B. subtilis* 168 as a reporter for the fatty acid synthesis pathway using firefly luciferase as a reporter. Similarly, Hutter *et al.* (2004) also

used *B. subtilis* 168 and the luciferase reporter to study a panel of reporter strains developed for different mode of actions. Urban *et al.* (2007) used a non-spore forming derivative of *B. subtilis* 168 (1S34) for their study of a panel of *Bacillus* reporters with firefly luciferase. The species also has different responses to different stimuli which further justify its selection as a model organism for mode of action reporter strain constructs (Urban *et al.*, 2007).

3.1.7 Titration for chitosan preparation

Titration is a basic chemistry technique which can be used to calculate the volume of an alkali or acidic substance to neutralise its opposite. Titration has been used to determine the degree of acetylation of chitosan, and it can also be used to determine the quantity of free acid remaining in a chitosan solution (Rusu-Balaita, Desbrières J. and Rinaudo, 2003). Chitosan behaves as a weak base therefore when dissolved in a strong acid, the slope of the titration curve can allow differentiation between protonation of the chitosan amine groups and free protons in solution. This permits confirmation of the calculation of the stoichiometric solution for chitosan.

Achieving a stoichiometric solution with minimal free protons in the solution allows a more accurate representation of the activity of chitosan, as opposed to the effect that the acid is achieving. Further to this, using an appropriate background control is essential for properly understanding the action that is being studied.

3.1.8 Chitosan antimicrobials in studies

Disc diffusion and agar diffusion studies are commonly used in chitosan studies and provide an illustration of chitosan's antimicrobial activity. However, broth inoculation studies with chitosan have been limited.

Oh *et al.* (2001) compared the antimicrobial activity of chitosans to spoilage organisms associated with mayonnaise including *Lactobacillus* and *Serratia* species. They used well characterised chitosans ranging in viscosity average molecular weights (MW) from 12 to 59 kDa and ranging in degree of deacetylation (DDA) from 75 to 87%. They studied the effects by broth incubation over time with viable cell counts and found that MW had a significant effect on the antimicrobial activity with species specific effects and concluded that chitosan

may be useful for increasing the shelf life of foods. But control details were not disclosed in the article.

Helander *et al.* (2001) compared two chitosans that were characterised only by their DDAs of 83 and 85% against several gram negative bacteria. They gave good detail to the preparation of the chitosan solutions but noted that definitive antimicrobial activities for species are difficult to ascertain due to the range of factors involved.

Younes *et al.* (2014) examined well chemically characterised chitosans against different species of gram positive and negative bacteria and fungi using agar well diffusion. They found that the chitosans they screened did not inhibit *P. aeruginosa* ATCC 27853 or *E. faecalis* ATCC 29212. They also broadly concluded that the inhibitory effect increased when chitosans were more deacetylated. The details of the chitosan sample preparation were limited though and they emphasised a pH effect but with limited sample preparation details.

Tikhonov *et al.* (2006) also compared chitosan with a viscosity average molecular weight of 300 kDa and DDA of 82% against gram negative and positive bacteria. They examined chitosans activity against gram positive and negative bacteria as well as fungi. They found that chitosan limited the growth of the fungi and bacteria species tested.

3.1.9 Mode of action

The mode of action of chitosans has yet to be fully confirmed. Due to inconsistencies in the way that studies are reported it remains difficult to achieve consensus.

In gram positive bacteria, Raafat *et al.* (2008) studied the mode of action of chitosan against *S. aureus* with transcriptional response data and found that the bacterial cell membrane became impaired but remained intact. They found changes in the expression profiles of several genes associated with stress regulation, autolysis and energy metabolism, leading them to suggest that chitosans mode of actions is related to interactions with teichoic acid in the cell wall and potential extraction of membrane lipids, which may subsequently lead to bacterial cell death. Raafat *et al.* (2008) further discussed how the applications of chitosan may relate to its mode of action including chitosans fat binding properties and hypothesised that chitosan may be able to extract lipids from the cell membrane.

When exposing chitosans to gram negative bacteria, Helander *et al.* (2001) observed vesicular structures on the outer membrane of the bacteria giving the appearance of a thicker cell envelope and suggested similarities to the action of PEI. Furthermore, Vila-Sanjurjo *et al.* (2019) highlighted quorum quenching activity from chitosan nanoparticles which may impact the growth of certain species and limit cell-cell communication.

3.1.10 Aims and objectives

Chitosan studies remain limited in many ways. Often, controls are not representative of the background conditions for solubilising the chitosan which make the results difficult to compare. Therefore, the aim of this chapter of work was to build on the previous characterisation of the chitosan produced from BSF to see if we can replicate the previously reported antimicrobial activity against certain foodborne bacteria and provide a general direction for application of the chitosan as an active biobased food contact material. It was important to focus on ruling out the effect that the acidic solution used to dissolve the chitosan had and highlight how different preparation methods can lead to different results, emphasising the importance of disclosing sample preparation in research articles so as to minimise conflicting conclusions. Finally, we wanted to study whether a panel of *Bacillus* reporter strains available can help in identifying a general pathway for a mode of antimicrobial activity.

The **aims** and objectives for this chapter are further summarised:

3 Assess the antimicrobial activity of chitosan

3.1 Develop antimicrobial susceptibility tests to *Escherichia coli* and S*taphylococcus aureus*

3.2 Measure the antimicrobial activity of chitosan to *Bacillus subtilis*

3.3 Measure the antimicrobial of chitosan against a selection of foodborne bacteria

3.4 Compare the responses of a panel of *Bacillus* reporter strains to different chitosans

3.2 Materials and Methods

3.2.1 General materials

3.2.1.1 Chemicals and consumables

90 mm Petri dishes, plastic spreaders and loops - Fisher Scientific, UK

Corning bioassay plates - Sigma Aldrich, Germany

Maximum recovery diluent, 9 mL and powder - Fisher Scientific, UK

Tryptone soya agar - Fisher Scientific, UK

Muller Hinton agar - Fisher Scientific, UK

Fraser broth - Fisher Scientific, UK

Bolton broth - Fisher Scientific, UK

Nutrient agar - Fisher Scientific, UK

Nutrient broth - Fisher Scientific, UK

Defibrinated horse blood - Fisher Scientific, UK

Sodium hydroxide pellets - Fisher Scientific, UK

Glacial acetic acid – Fisher Scientific, UK

6 M Hydrochloric acid – Sigma Aldrich, Germany

Sodium acetate - Fisher Scientific, UK

Chloramphenicol - Fisher Scientific, UK

3.2.1.2 Equipment

SevenEasy pH meter - Mettler Toledo

Vortex - Fisherbrand Whirlmixer – Fisher Scientific, UK

Balance (0.1 mg) - Sartorius, Germany

Edwards SuperModulyo Freeze Dryer

Water bath - Grant Instruments UK

3.2.1.3 Chitosans and microbiological

Nutrient broth, nutrient agar and Mueller Hinton agar were purchased from Fisher Scientific, UK. Chitosan samples were obtained from Glentham Life Sciences and generated in lab from BSF larvae as described in Chapter 2. Characteristics of the chitosans are summarised in Table 11.

Table 11 – Chitosan characteristics from Chapter 2. DDA = degree of deacetylation. MW AF4 = Average molecular weight from asymmetric flow field flow fractionation with multi angle light scattering and refractive index measurements. [n] = intrinsic viscosity. MM = molar mass. Da = Dalton.

	DDA (%)	**MW AF4 (g mol^{-1})**	**[n] (mL g^{-1}) from viscometry**	**MM from [n] (Da)**
NB 5cps	87.40		37.35	27194.82
10cps	87.67	50000 ± 4.5%	82.05	49983.12
Sq	93.39	220000 ± 0.9%	796.45	289885.33
BSF	96.63	130000 ± 3.1%	490.45	199240.31

Corning square bioassay dishes (245 mm) were used for agar well diffusion assays. Standard Petri dishes (90 mm) were used for reporter strains and against the different bacterial species. Campygens and defibrinated horse blood were purchased from Fisher Scientific, UK. A 5 mm hole borer was used to aseptically cut wells in the agar plates.

Chloramphenicol positive controls were made up, diluted to concentrations and stated for each experimental phase. *Escherichia coli* ATCC 25922; *Salmonella cerro* NCTC 5801; *Bacillus subtilis* NCTC 5398; *Enterococcus faecalis* ATCC 29212; *Pseudomonas aeruginosa* ATCC 10332; *Listeria monocytogenes* NCTC 5214; *Campylobacter jejuni* ATCC 11168; *Staphylococcus aureus* ATCC 29213 were used from frozen stocks maintained at Fera Science Ltd.

Multiple experiments of agar well diffusion were performed to optimise the sample preparation to display inhibition. Then these samples were applied across the range of different foodborne bacteria as described above.

3.2.2 pH titration

Stock 2 M solutions of HAc, HCl and NaOH were diluted accordingly and used to dissolve set quantities of chitosan. Chitosan was dissolved at different concentrations in 0.02 M HCl and allowed to dissolve overnight at 4 ^{o}C (refrigerated). The solution was then titrated with dilute 0.02 M NaOH into chitosan/acid solutions with magnetic stirring and pH measured at set volumes titrated to produce titration curves. 2.5 g L^{-1}; 5 g L^{-1} of chitosan was dissolved in 0.02 M HCl for comparison of how much 0.02 M HCL is still available when used to dissolve different quantities of chitosan.

3.2.3 Chitosan samples

3.2.3.1 Agar well diffusion

3.2.3.1.1 *Escherichia coli* and *Staphylococcus aureus*

Initially 16 samples of varying concentrations of chitosans (Table 12) with different viscosities were assessed against gram negative *E. coli* and gram positive *S. aureus*. This allowed comparisons of different molecular weight samples to determine if there was a pattern. Three replicates were performed per sample, means and standard deviations for measured diameters of zones of inhibition were calculated.

Table 12 – Sample details for initial agar well diffusion against *E. coli* ATCC 25922 and *S. aureus* ATCC 29213. HAc = Acetic acid. ChlorA = chloramphenicol.

Tube no.	Sample	HAc (M)	Chitosan (g L^{-1})
1	HAc	0.1740	0
2	5cps	0.1392	8
3	5cps	0.2088	12
4	5cps	0.2784	16
5	10cps	0.1392	8
6	10cps	0.2088	12
7	10cps	0.2784	16
8	Squid	0.1392	8
9	Squid	0.2088	12
10	Squid	0.2784	16
11	MMW	0.1392	8
12	BSF	0.1392	8
13	BSF	0.2088	12
14	BSF	0.2784	16
15	ChlorA 0.1 g L^{-1}	0.0000	0
16	ChlorA 1.0 g L^{-1}	0.0000	0

3.2.3.1.2 *Escherichia coli*

Further optimisation of samples was then conducted with another nine samples (Table 13) against *E. coli*. Low viscosity chitosans were used due to the ease of pipetting and increased chitosan load. Three replicates were performed per sample, means and standard deviations were then calculated.

Table 13 – Samples compositions used for second agar well diffusion against *E. coli* ATCC 25922.

Sample		Chitosan (g L^{-1})	HCl (M)
1	HCl control	0	0.10
2	HCl control	0	0.15
3	HCl control	0	0.20
4	5cps	15	0.10
5	5cps	25	0.15
6	5cps	35	0.20
7	10cps	15	0.10
8	10cps	25	0.15
9	10cps	35	0.20

3.2.3.1.3 *Bacillus subtilis*

Eight samples (Table 14) were then screened against *B. subtilis*. 5cps chitosan was used for optimising inhibition to use as a positive control chitosan (absolute strongest concentration) and checking samples for inhibition for use against reporter strains. Four replicates were performed per sample with means and standard deviations calculated.

Table 14 – Sample compositions which were used for agar well diffusion antimicrobial susceptibility testing against *B. subtilis* NCTC 5398.

Sample	HCl (M)	Chitosan (g L^{-1})	NaCl (M)
1	0.2	35	0.0
2	0.0	0	0.2
3	0.0	0	0.1
4	0.2	0	0.0
5	0.1	15	0.0
6	0.2	35	0.2
7	0.4	35	0.0
8	0.2	0	0.2

3.2.4 Versus different species

For further screening against different bacterial species four samples were selected.

1 = negative control - pH adjusted HCl control (pH 4.67) HCl/NaOH

2 = BSF 10 g L^{-1} (pH 5.18)

3 = Sq 10 g L^{-1} (pH 4.99)

4 = 5cps 35 g L^{-1} (pH 4.69)

3.2.4.1 Culture

Frozen stock bead (stored at -80 ^{o}C) was defrosted and aseptically added into a 10 mL starter broth culture (nutrient broth for non-fastidious, Bolton broth for *Campylobacter*, Fraser broth for *Listeria*) for 72 hours at 37 ^{o}C. 72 hour cultures were then streaked for purity and

10 µL of 72 h broth was aseptically transferred into fresh 10 mL nutrient broth for 18-20 h at 37 ^{o}C.

3.2.4.2 Chitosan versus different species and reporter strains

20 hour cultures were grown in broth overnight at 37 ^{o}C then diluted to 10^7. After that 100 µL of diluted broth was added to 100 mL agar and used to pour four plates per species. *Campylobacter* and *Listeria* agars were supplemented with defibrinated horse blood (5 mL per 100 mL).

3.2.4.3 Set up

Muller Hinton agar was prepared and autoclaved in advance. When required this was melted in a steamer for two hours, then allowed to temper in a water bath at 45 ^{o}C for 40 minutes. 1 mL of 20 h culture was added into a 9 mL MRD universal and diluted further where necessary to achieve 10^7. 100 µL of 10^7 bacterial solution was then added to 150 mL Muller Hinton agar, mixed and pour plated into a square bioassay dish. This was then allowed to solidify for 20 minutes at room temperature.

Holes were then aseptically bored using ethanol, Bunsen burner and a 5 mm hole borer. Samples pipetted into the wells on the plate. Plates were incubated at 37 ^{o}C overnight, except *Campylobacter*, which was incubated for 48 hours.

Zones of inhibition were measured as diameters in mm using a digital calliper. Calculation of well sizes was averaged across measurements throughout experimentation.

3.2.5 Reporter strains

Five reporter strains of *B. subtilis* strain 1S34 were provided by Dr. Jem Stach, Newcastle University and used to screen chitosans against different mechanisms of action. They were produced according to Urban *et al.* (2007) except with a minor modification, as β-galactosidase was used as the reporter gene (Dr Jem Stach, personal communication).

YvqI, YjaX, YpuA, YvgS and DinB strains were used. YvqI highlights antibiotics with cell wall activity. As a positive control bacitracin was used. YjaX highlights antibiotics with activity against fatty acid synthesis leading to fatty acid synthesis inhibition (Fischer *et al.*, 2004). As a

positive control triclosan was used for this strain. YpuA highlights antibiotics that direct stress onto the cell wall and envelope. Cefoxitin was used as a positive control for this strain. YvgS highlights antibiotics that inhibit RNA synthesis. Rifampicin was used as a positive control for this strain. DinB highlights antibiotics that cause DNA damage. Nalidixic acid was used as a positive control.

Five samples including positive controls were applied to each plate:

1 = pH adjusted HCl control (pH 4.67)

2 = BSF chitosan 10 g L^{-1} (pH 5.18)

3 = Sq chitosan 10 g L^{-1} (pH 4.99)

4 = 5cps chitosan 35 g L^{-1} (pH 4.69)

5 = Positive control specific to each reporter strain (detailed above).

3.2.5.1 Culture preparation

Frozen stocks of each strain were applied into starter cultures (5 mL shaking overnight at 37 ^{o}C). Then 5 mL of starter culture into 45 mL nutrient broth with X-Gal (5-bromo-4-chloro-3-indoyl-β-ᴅ-galacto-pyranoside) (0.25% (v/v)), isopropylthio-β-galactoside (IPTG) (0.05% (v/v) for YjaX strain only) and antibiotic (0.1% (v/v); erythromycin for YvqI, YvgS, YpuA, YjaX; chloramphenicol for DinB). Then added to 150 mL nutrient agar and stored in a water bath at 50 ^{o}C to temper. Plates were poured following gentle mixing.

3.2.5.2 Experimental setup

Four plates per strain were prepared. Two plates were used for disc diffusion and two plates for agar well diffusion in 90 mm petri dishes. To prepare the plates with the samples a 5 mm hole borer was used with a Bunsen burner following aseptic technique. Discs were dipped into solutions and plated. For agar well diffusion assays 50 µL of each sample was pipetted into wells made by the hole borer. Plates were incubated overnight at 37 ^{o}C and a positive result was identified by a blue ring around the sample.

3.3 Results

3.3.1 Titration

Titration allowed calculation of how much acid was needed to dissolve chitosan without there being a large amount in excess which could impact antimicrobial effects. From the initial titration curves, it could first be concluded that there were two clear inflexion points for the neutralisation of chitosan dissolved in hydrochloric acid (Figure 35). This suggested that chitosans amine associated protons were neutralised after the free hydrochloric acid protons. Therefore, concentrating on chitosan dissolved in strong acid made it more clear to conclude the volume of acid needed to dissolve chitosan.

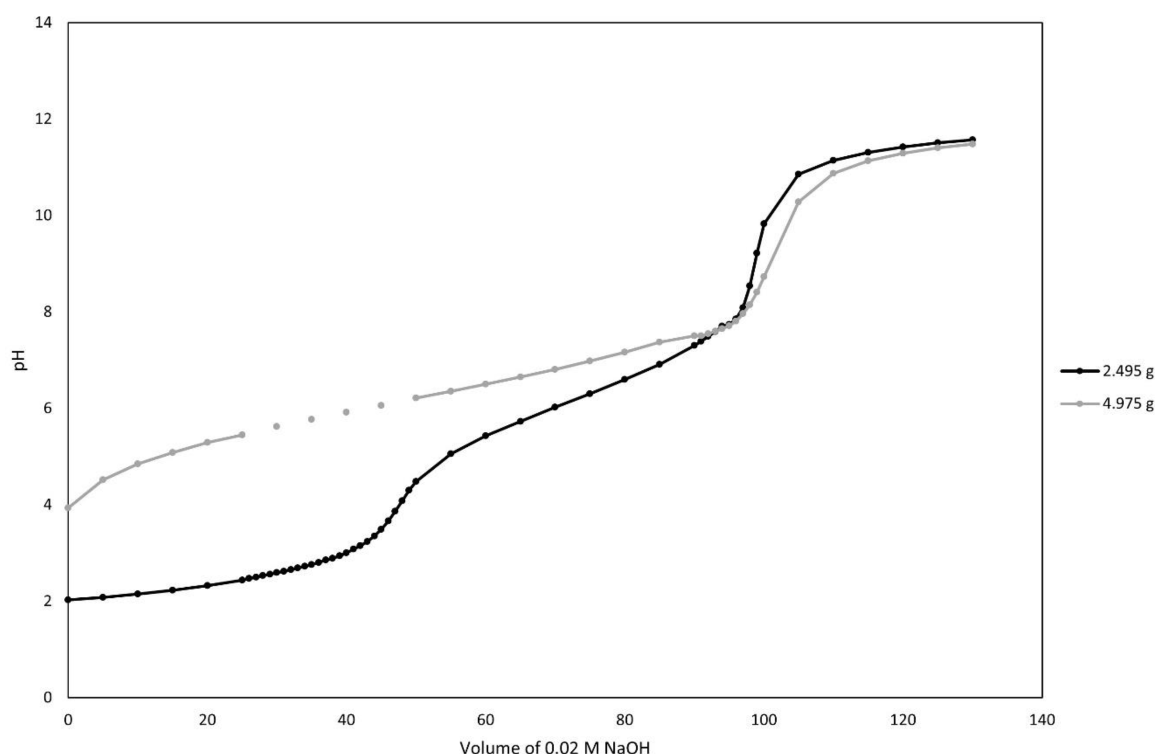


Figure 35 – Titration curve for different concentrations of 10cps chitosan (2.495 g L^{-1} and 4.975 g L^{-1}) dissolved in 100 mL of 0.02 M HCl titrated with 0.02 M NaOH.

From the titration curves it could then be interpreted that the first inflexion point, highlighting the point in which the excess strong acid had been neutralised and protons associated with the amine group of the deacetylated chitosan, was moving to the left as the concentration of chitosan increased. This means that there were fewer free protons in solution and we were closer towards achieving stoichiometry. It also highlighted the pH value

(~5) which chitosan begins to be neutralised, where the plateau following the inflexion point proceeds.

From these titration experiments and through calculation of the average monomer composition of the chitosan from ^{1}H NMR DDA calculation, we could estimate the amount of acid needed to dissolve chitosan with minimal free acid in the solution. The value calculated for this was 3.67 g L^{-1} in 0.02 M HCl. For further experiments (from Section 3.3.2.2 onwards) this was rounded to 3.5 g of chitosan dissolved in 0.02 M HCl.

3.3.2 Agar well diffusion optimisation

3.3.2.1 – *Escherichia coli* and *Staphylococcus aureus*

Firstly several chitosan samples were prepared which allowed the comparison of different concentrations, molecular weights and degrees of acetylation (Table 15). Inhibition was evident for both species of bacteria for negative and positive controls as well as chitosan samples (Figure 36). Chitosan samples produced a white colouration of the agar. High viscosity samples were difficult to apply and limited experimentation.

Table 15- Sample compositions used for agar well diffusion antimicrobial susceptibility testing against *E. coli* ATCC 25922 and *S. aureus* ATCC 29213. Chitosans used were previously characterised by ^{1}H NMR for degree of acetylation and molecular weight by asymmetric flow field flow fractionation with multi angle light scattering and refractive index measurements and capillary viscometry.

Tube no.	Sample	HAc (M)	Chitosan (g L^{-1})
1	HAc	0.1740	0
2	5cps	0.1392	8
3	5cps	0.2088	12
4	5cps	0.2784	16
5	10cps	0.1392	8
6	10cps	0.2088	12
7	10cps	0.2784	16
8	Squid	0.1392	8
9	Squid	0.2088	12
10	Squid	0.2784	16
11	MMW	0.1392	8
12	BSF	0.1392	8
13	BSF	0.2088	12
14	BSF	0.2784	16
15	ChlorA 0.1 g L^{-1}	0.0000	0
16	ChlorA 1.0 g L^{-1}	0.0000	0

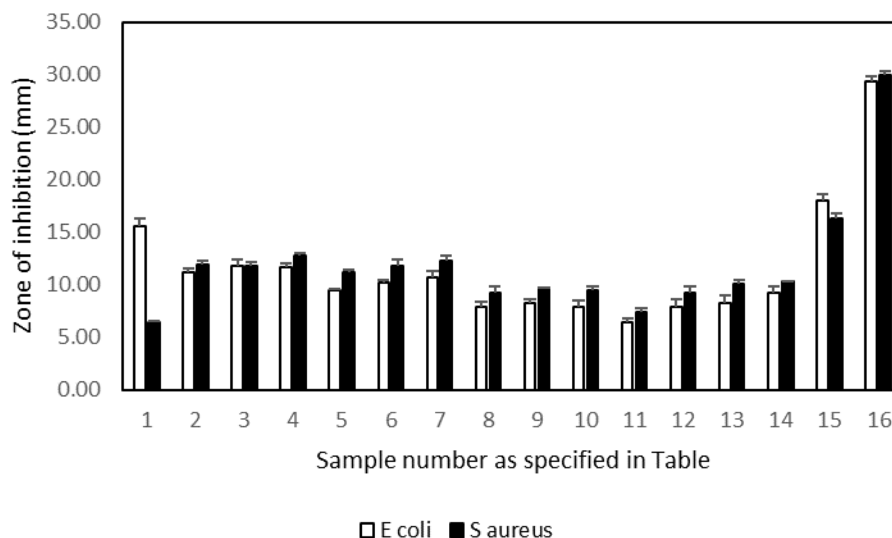


Figure 36 – Mean (± S.D.) diameters of the zones of inhibition of different chitosan samples (Table 15) against *E. coli* ATCC 25922 and *S. aureus* ATCC 29213.

3.3.2.1.1 *Escherichia coli*

The negative control had a higher inhibition than chitosan samples but remained lower than the positive controls (Chloramphenicol). The negative control had a mean zone of inhibition of 15.58 ± 0.75 mm. The 0.1 g L^{-1} and 1 g L^{-1} chloramphenicol controls had mean zones of inhibition of 17.94 ± 0.71 mm and 29.37 ± 0.48 mm respectively. The chitosan sample which produced the largest zone of inhibition for *E. coli* was sample 3 with a mean zone of inhibition of 11.83 ± 0.61 mm.

Chitosan samples showed larger zones of inhibition for lower viscosity samples. The lowest viscosity chitosan sample (5cps) had the largest mean zone of inhibition for sample 3 with a mean zone of inhibition of 11.83 ± 0.61 mm. The 10cps chitosan which has a slightly higher viscosity had smaller zones of inhibition. Sample 7 had the largest mean zone of inhibition with 10.66 ± 0.61 mm. With a more viscous sample than both the 5cps and 10cps, the squid chitosan had smaller zones of inhibition. The largest zone of inhibition was for sample 9 with a mean zone of inhibition of 8.27 ± 0.29 mm.

The chitosan produced from the black soldier fly produced zones of inhibition which fell between the squid chitosan and the 10cps chitosan with sample 14 producing the largest zone of inhibition with a mean of 9.25 ± 0.59 mm.

3.3.2.1.2 *Staphylococcus aureus*

The negative control had less of an inhibitory effect on *S. aureus* than *E. coli*. The negative control produced a mean zone of inhibition of 6.44 ± 0.12 mm. The 0.1 g L^{-1} and 1 g L^{-1} chloramphenicol controls produced zones of inhibition of 16.33 ± 0.49 mm and 29.90 ± 0.37 mm respectively.

It could be concluded that chitosan samples had an inhibitory effect on *S. aureus* because all samples produced zones of inhibition greater than the negative control. Similarly to *E. coli*, *S. aureus* showed slightly greater diameters of the zones of inhibition for lower viscosity samples. The largest mean zone of inhibition was for sample 4 which was the lowest viscosity chitosan with a mean zone of inhibition of 12.75 ± 0.26 mm. The 10cps then followed the 5cps with the next largest mean zone of inhibition for sample 7 of 12.31 ± 0.42 mm. The squid chitosan which was of a higher viscosity than both the 5cps and 10cps followed the same pattern as for the *E. coli* with lower mean zones of inhibition compared to 5cps and

10cps chitosans. Sample 9 produced the largest mean zone of inhibition for the squid derived chitosan with a mean zone of inhibition of 9.52 ± 0.19 mm.

As with *E. coli* the chitosan produced from the black soldier fly produced zones of inhibition which were larger than the squid chitosan but smaller than the 5 and 10cps chitosans. Sample 14 produced the greatest zone of inhibition for the BSF chitosan with a mean zone of inhibition of 10.26 ± 0.08 mm.

3.3.2.2 *Escherichia coli*

From the first experiment it was evident that the negative control was producing a greater inhibitory effect than chitosan samples against *E. coli*. Therefore further focus was directed to *E. coli* to clarify whether chitosan does in fact have inhibitory effects or whether it is the acid used to dissolve chitosan that is causing inhibition.

Table 16 – Samples compositions for agar well diffusion against *E. coli* ATCC 25922.

Sample		Chitosan ($g\ L^{-1}$)	HCl (M)
1	HCl control	0	0.10
2	HCl control	0	0.15
3	HCl control	0	0.20
4	5cps	15	0.10
5	5cps	25	0.15
6	5cps	35	0.20
7	10cps	15	0.10
8	10cps	25	0.15
9	10cps	35	0.20

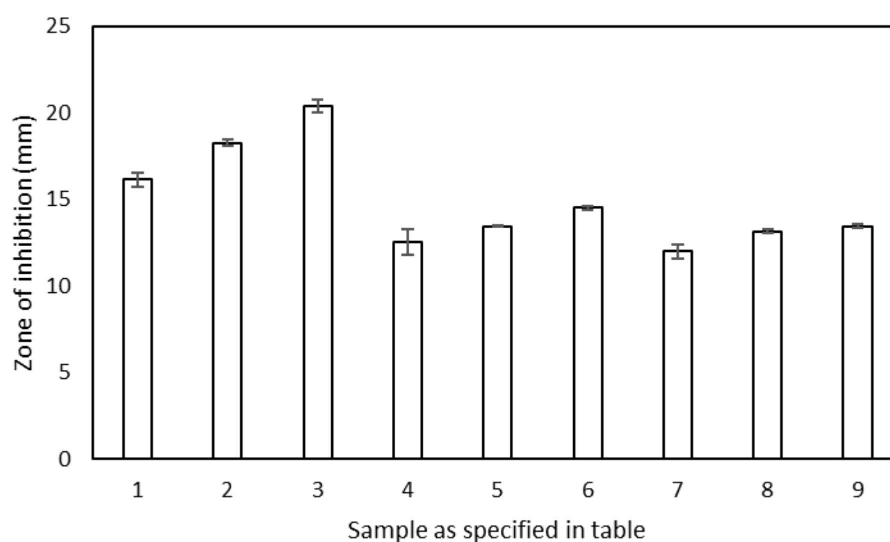


Figure 37 – Mean (± S.D) diameters of zones of inhibition for increasing concentrations of hydrochloric acid along with increasing concentrations of chitosan (Table 16) using agar well diffusion with *E. coli* ATCC 25922.

Table 16 shows the sample compositions used. Figure 37 depicts the inhibition of *E. coli* with different concentrations of hydrochloric acid and increasing chitosan concentrations in the same concentrations of hydrochloric acid. The largest mean zone of inhibition was produced for the highest molarity of HCl (0.2 M) with sample 3. Sample 3 had a mean zone of inhibition of 20.40 mm and a standard deviation of 0.36 mm. The lowest concentration of HCl (sample 1) had a mean zone of inhibition of 16.16 mm and a standard deviation of 0.41 mm. The lower viscosity chitosan (5cps) had its highest inhibitory effect at its highest concentration (sample 6) which had a mean zone of inhibition of 14.52 mm and a standard deviation of 0.12 mm. The higher viscosity chitosan (10cps) had a lower inhibitory effect than the 5cps chitosan with its highest inhibitory sample (sample 9) producing a mean zone of inhibition of 13.46 mm and a standard deviation of 0.098 mm.

It is clear that the samples not containing chitosan are measuring greater inhibition than the samples containing chitosan. But also the pattern of the increase in hydrochloric acid among samples and the increase in chitosan concentration may evidence a dampening effect of the acid's inhibition.

3.3.2.3 *Bacillus subtilis*

Gram positive *B. subtilis* was then screened to ascertain whether it is susceptible to chitosan samples (Table 17). Sodium chloride was added to some samples to determine whether salt concentration, as a result of the use of the hydrochloric acid and sodium hydroxide to balance pH, was affecting the inhibition. This would allow further investigation with *Bacillus* reporter strains. Calculation of well sizes was averaged across 18 diameter measurements throughout experimentation. Mean well size diameter 5.51 mm.

Table 17 – Sample compositions used for agar well diffusion antimicrobial susceptibility screening against *B. subtilis* NCTC 5398.

Sample	HCl (M)	Chitosan ($g\ L^{-1}$)	NaCl (M)
1	0.2	35	0.0
2	0.0	0	0.2
3	0.0	0	0.1
4	0.2	0	0.0
5	0.1	15	0.0
6	0.2	35	0.2
7	0.4	35	0.0
8	0.2	0	0.2

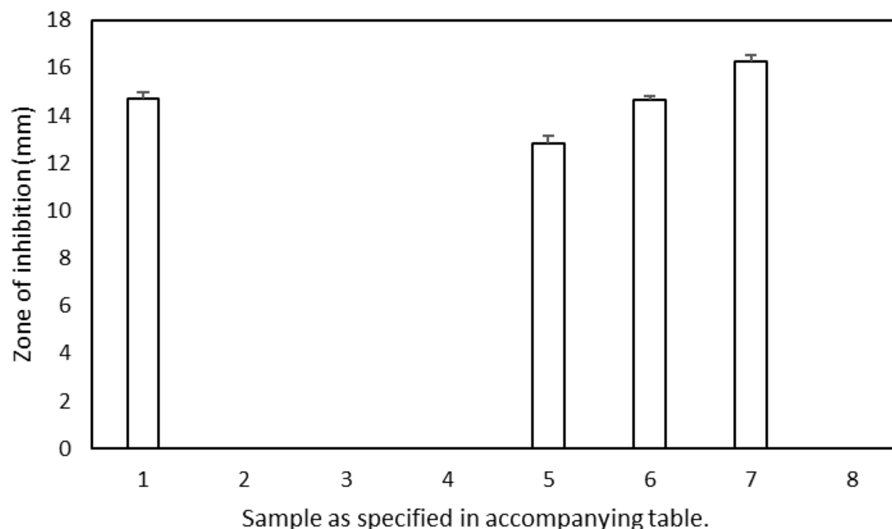


Figure 38 – Mean (± S.D.) diameter of zones of inhibition for *B. subtilis* NCTC 5398 with varying sample compositions specified in Table 17.

Interestingly the 0.2 M hydrochloric acid used here without chitosan did not inhibit this strain and a zone of inhibition could not be measured (Figure 38). This appears to show the opposite effect to the previous experiment in which inhibition was greater in samples without chitosan. Whereas here, only samples containing chitosan exhibited visible inhibition. These results and those from Section 3.3.2.1 imply that gram positive bacteria may be less susceptible to acid but more susceptible to chitosan than gram negative bacteria.

Samples 1 and 6 showed similar inhibition. Sample 1 had a mean zone of inhibition of 14.69 mm with a standard deviation of 0.28 mm, while sample 6 had a mean zone of inhibition of 14.63 mm and a standard deviation of 0.16 mm. This suggests that the addition of NaCl did not significantly impact the antimicrobial activity.

The excess hydrochloric acid applied to sample 7 increased the inhibition with a mean zone of inhibition of 16.23 mm and a standard deviation of 0.32 mm. This evidences that the way chitosan samples are prepared can have an impact on the results achieved in antimicrobial susceptibility testing.

3.3.3 Versus different species:

The positive and negative controls worked well (Table 18). Chitosan failed to produce a zone of inhibition for *E. faecalis*. *B. subtilis* proved to be the most sensitive, producing a visible zone of inhibition for a weaker concentration of chitosan (SqCS 10 g L^{-1}, sample 3). *S. cerro, P. aeruginosa* and *E. coli* had inhibition for chitosan sample number 4, the highest concentrated chitosan, but were not inhibited by weaker concentrations of chitosans. *P. aeruginosa* had a green colour appear around the zone of inhibition. *Campylobacter* and *Listeria* were difficult to distinguish due to the way the blood aggregated, possibly could be due to the chitosan sample.

Table 18 – Inhibition detected for different bacterial species against different samples tested. 1 = negative control- pH adjusted /HCl control (pH 4.67) HCl/NaOH, 2 = BSF 10 g L^{-1} (pH 5.18), 3 = Sq 10 g L^{-1} (pH 4.99), 4 = 5cps 35 g L^{-1} (pH 4.69). '+' = inhibition zone visible, '-' = inhibition zone not visible.

	1	**2**	**3**	**4**
Escherichia coli ATCC 25922	-	-	-	+
Bacillus subtilis NCTC 5398	-	-	+	+
Salmonella cerro NCTC 5801	-	-	-	+
Enterococcus faecalis ATCC 29212	-	-	-	-
Pseudomonas aeruginosa ATCC 10332	-	-	-	+

The weaker chitosan sample from black soldier fly (BSF 10 g L^{-1}, sample 2) did not produce visible inhibition for any species even though it was at a similar concentration as sample 3. This highlights how source and differences in properties such as molar mass distribution and degree of deacetylation could be having an impact on the antimicrobial effect. It also highlights how easy it is to get results that conflict with one another when characteristics of chitosan are not disclosed in research articles.

3.3.4 Bacillus reporter strains

For this experiment two plates were performed with disc diffusion and two with agar wells for each reporter strain. The positive controls produced visible zones of inhibition for all five reporter strains and produced positive blue responses (Tables 19, 20). The background control produced no visible zones of inhibition. The three chitosan samples used all produced visible zones of inhibition for all five reporter strains.

Table 19 – Summary of the inhibition of the reporter strains to the samples used. '+' = visual inhibition zone, '-' = no visual inhibition. 1 – pH adjusted HCl control (pH 4.67). 2 – BSF chitosan 10 g L^{-1} (pH 5.18). 3 – Sq chitosan 10 g L^{-1} (pH 4.99). 4 – 5cps chitosan 35 g L^{-1} (pH 4.69). 5 – Positive control specific to each reporter strain.

	1	2	3	4	5
YvgS	-	+	+	+	+
YpuA	-	+	+	+	+
YvqI	-	+	+	+	+
DinB	-	+	+	+	+
YjaX	-	+	+	+	+

Table 20 - Summary of the responses of the reporter strains to the samples used. '+' = blue coloured ring present, '-' = no colour. 1 – pH adjusted HCl control (pH 4.67). 2 – BSF chitosan 10 g L^{-1} (pH 5.18). 3 – Sq chitosan 10 g L^{-1} (pH 4.99). 4 – 5cps chitosan 35 g L^{-1} (pH 4.69). 5 – Positive control specific to each reporter strain.

	1	2	3	4	5
YvgS	-	-	-	-	+
YpuA	-	-	-	-	+
YvqI	-	-	-	-	+
DinB	+	-	-	-	+
YjaX	-	+	+	-	+

The background control interestingly produced a blue response to the DinB reporter strain at the edges of the agar well and the edges of the discs. There was no blue (positive) response to the chitosan samples for the DinB reporter despite the negative control producing one itself. The YjaX reporter strain produced a positive response to two of the chitosan samples indicating a possible link to a mode of action or stressor. It was also noted that the X-Gal exposure produced a blue colour to the whole agar for DinB and YjaX which may have implications for the positive responses seen.

3.4 Discussion

Here several chitosans previously characterised by ^{1}H NMR and AF4-MALS-RI, defining their average DA and molar mass distributions, were compared against each other, against a selection of bacteria and *Bacillus* reporter strains. From these investigations several conclusions could be made.

3.4.1 Antimicrobial susceptibility testing

3.4.1.1 Acid /pH effects

Initially, it became apparent that there were differences in the way each strain of bacteria responded to chitosans. Some were more inhibited by the background control which led to the suggestion that an acid effect may be misinterpreted to falsely conclude that chitosan is having a significant antimicrobial effect, when in fact it is the acid (used to dissolve the chitosan) that is producing the inhibitory zones. This is particularly concerning when publications do not include suitable background controls when analysing the results. Therefore significant caution is needed when drawing conclusions from such research articles.

In the comparison of the first experiment where *E. coli* and *S. aureus* had zones of inhibition measured it was clear that *E. coli* was being inhibited more by acid alone than when chitosan was dissolved in an equal concentration of the same acid. This suggests that the free acidic ions are more effective than when they protonate the free amine groups of the D-glucosamine sugars. Therefore it was necessary to ensure minimal free acid protons in the samples using titration and stoichiometry. Once the free acid protons had been corrected and we applied the stoichiometric amounts of acid, the background controls were effective in showing no inhibition. An alternative approach may be to try to use other weaker acids to dissolve chitosan that may have limited inhibition against target organisms.

Other previous studies have highlighted the importance of pH when studying chitosan's activity. Younes *et al.* (2014) used pH 5.5 for their samples at 25 g L^{-1} . They also examined the pH effect between 4.4 and 6.1 with viable cell counts. They found viable cell counts to be greater at higher pH and counts decrease as pH decreased. Jing *et al.* (2007) found similar results for their pH effects. They measured pH effects separately to their main antimicrobial activity. In the main antimicrobial activity study they used chitosan at 1% (w/v) in 0.5% (v/v)

acetic acid. They did not mention the pH at which they dissolved their main samples which produced inhibitory zones. But during the pH study they found that increasing the pH in a graduated manner from 4 up to 6 reduced the size of the zones of inhibition.

3.4.1.2 Effect of chitosan properties and quantifying measurements

The viscosity of the samples limited their concentrations when applying them to plates. It was evident that chitosans with a higher average molar mass and reported viscosity were resulting in smaller zones of inhibition. This could be due to the ability of larger molecules to diffuse, which may also be impacting the true result. For example larger molar mass samples may not diffuse significantly enough to produce a zone of inhibition which is visually detectable. This makes the agar well diffusion limited for quantifying chitosans antimicrobial capability.

However, in comparison to Younes *et al.* (2014), who compared chitosans with varying characteristics it was evident that chitosans with the same acetylation but with slightly differing molar masses produced similar zones of inhibition. Their data actually highlighted the degree of acetylation to be a slightly more impactful characteristic than molar mass. Younes *et al.* (2014) also highlighted that more highly acetylated chitosans were not inhibiting bacterial growth as well as highly deacetylated chitosans. This would likely be due to the charge density on the molecules, as chitosans activities are associated with its cationically charged amine group. They used chitosan samples at pH 5.5 which will have had some impact on the protonation and solubility of the samples too. Jing *et al.* (2007) also looked at differences in inhibition with different molecular weight chitosans and their data suggested a general trend of decreasing molecular weight with increasing zone of inhibition diameter. This is similar to what we have found.

3.4.1.3 Previous studies

A fully characterised chitosan derived from *Hermetia illucens* was characterised for its antimicrobial activity against the different species tested. There are limited insect chitosan studies which have examined antimicrobial activity with suitable controls.

Source has previously been considered to impact the activity of chitosan. However, this is most likely related to the characteristics of the chitosan produced rather than specifically the

biological source. Different sources will need different extraction methods to remove all contaminants. If not fully purified, residual molecules will impact further purification and deacetylation. Therefore, if chitosan characteristics are fully reported with the study, comparisons can be made.

E. faecalis ATCC 29212 was not inhibited by any of the chitosans. Younes *et al.* (2014) also failed to inhibit the same strain of *E. faecalis* with the chitosans they tested. *E. faecalis* is a bacterium found in the intestinal tract of humans and the genus is known to be able to survive harsh conditions. This is interesting from the perspective that beneficial bacteria in the gut of humans are not all inhibited by chitosan should it be consumed. Chitosan is known to be permitted to be used in foods in several countries (Baldrick, 2010).

Pseudomonas species are often associated with food spoilage (Bruckner *et al.*, 2012). They are able to grow at lower temperatures which means they are often associated with refrigerated foods such as milk and meats (Bellassi *et al.*, 2021; Bruckner *et al.*, 2012). We screened *P. aeruginosa* ATCC 10332 which showed inhibition to the chitosans used. Younes *et al.* (2014) and Jing *et al.* (2007) have both tested chitosans against *P. aeruginosa* ATCC 27853. Younes *et al.* (2014) reported that chitosans did not inhibit *P. aeruginosa*. However, Jing *et al.* (2007) found that chitosan did inhibit this strain.

Bacillus and *Escherichia* are genera with species which are known to cause food associated health issues (EC, 2005). Some of our chitosan samples inhibited *B. subtilis* NCTC 5398. Jing *et al.* (2007) used *B. subtilis* NCTC 9372 and also found that chitosan caused inhibition. *E. coli* ATCC 25922 produced zones of inhibition for both Jing *et al.* (2007) and Younes *et al.* (2014) when exposed to chitosans. This was also the case in our work.

Salmonella is a genus of bacteria associated with food poisoning illnesses from raw foods resulting in salmonellosis (FSA, 2018b). We screened *Salmonellla cerro* NCTC 5801 which also showed inhibition to the chitosan samples it was exposed to. Other studies have also tested chitosan to *Salmonella* species and found them also to be inhibited by chitosans. Younes *et al.* (2014) found *Salmonella typhi* was inhibited by chitosan samples which were highly deacetylated. Helander *et al.* (2001) exposed strains of *Salmonella typhimurium* to chitosans. One strain had more cationically charged complexes in its outer membrane. They found that this increase in cationic charges reduced the strains susceptibility to chitosan.

Campylobacter jejuni and *Listeria monocytogenes* were also tested. Due to their fastidious nature, they required agar supplementation with blood. However, due to chitosans haemostatic properties in which it coagulates blood, it was difficult to distinguish a zone of inhibition.

3.4.2 Reporter strains

The *Bacillus* reporter strains all showed inhibition zones for each of the three chitosan samples. This was different to *B. subtilis* strain NCTC 5398 which was not inhibited by the BSF 10 g L^{-1} sample . This may have been due to this sample having a slightly higher pH than the other samples.

The DinB and YjaX reporter strains had overexposure from X-Gal in which the positive control caused the whole agar plate to have a mild colour change, this made it harder to distinguish a positive response. However positive responses to other samples were still noticeable due to an increased colour density around the zones of inhibition compared with samples which did not have a reporter response.

The DinB reporter strain, which reports on antimicrobials that damage DNA, showed a positive response to the background control but did not show any response to the chitosan samples. While the YjaX reporter strain which highlights fatty acid synthesis inhibition showed a positive response to two chitosan samples but not the background control. Interestingly the two chitosan samples had lower concentrations than the third chitosan sample which did not show a positive response which may be indicative of an inverse concentration dependent activity. They also had higher molar masses, viscosities and degrees of deacetylation compared with the chitosan sample with the negative YjaX response which could indicate differences in the way bacteria respond to chitosan molecules with different characteristics. This may also relate to PEI selectivity to certain microbial taxa (Gibney *et al.*, 2012; Mikula *et al.*, 2018).

Chitosan's mode of action has previously been speculated on based on its characteristics. It has been associated with cell wall interactions in which it is suggested that chitosan may bind to teichoic acids in the cell wall (Raafat *et al.*, 2008; Verlee, Mincke and Stevens, 2017). It has also been reported that chitosan nanoparticles interrupt quorum sensing in *E. coli* and may induce multi peptide resistance factors in *S. aureus* (Raafat *et al.*, 2017; Vila-Sanjurjo *et al.*,

2020). No chitosan sample produced a positive response to either of the reporter strains related to cell wall interaction against *B. subtilis*, YpuA -cell envelope stress and cell wall stress, YvqI - cell wall active antibiotics.

Raafat *et al.* (2008) studied the effect of chitosan on *Staphylococcus* species. They found that chitosan did not cause cell wall lysis but found depolarisation of the cell membrane. They also noted that the cell membrane remained intact but it became more permeable to small intracellular components. They linked chitosans fat binding properties to a potential mode of action in suggesting that chitosan may be binding to teichoic acid and extracting lipopolysaccharides from the cell membrane.

In contrast, Helander *et al.* (2001) studied chitosan's interactions with the gram negative bacterial cell wall. They found that chitosan treatment did not result in the release of lipopolysaccharides or membrane lipids but made bacteria sensitive to the uptake of dyes. They highlighted vesicles using microscopy on the cell surface of chitosan treated bacteria which may result in the loss of barrier function. They also showed that reduced susceptibility to chitosan could be achieved through changes in the charges of the lipopolysaccharides in different *Salmonella typhimurium* strains.

Raafat *et al.* (2017) noted a similar response in gram positive bacterium *S. aureus* in which they found a chitosan resistant variant which possessed a lower charge of its cell wall and cell membrane, and cross resistance to cationic antimicrobial peptides. They also noted changes in metabolism such as increased membrane lipid (lysyl-phosphatidylglycerol, LPG) production. In their transcriptional analysis they highlighted changes in the gene expression profiles to increase production of components of the cell envelope. This included increased expression of genes for proteins associated with cell wall biosynthesis such as HMG-CoA (Hydroxymethylglutaryl-coenzyme A) synthase which they suggested may be to allow the chitosan resistant variant capability to synthesise more lipid carrier.

If chitosan is indeed inducing a change in metabolism in bacteria that includes the increase in cell wall biosynthesis then fatty acid synthesis may also be upregulated, though this needs further confirmation. This may mean that the positive response of the YjaX may also be due to the increased expression of the fatty acid synthesis pathway without it being inhibited.

YjaX codes for 3-oxoacyl-acyl carrier protein synthase III, an important enzyme in the fatty acid synthesis pathway (Fischer *et al.*, 2004), part of the family of β–keto-acyl-acyl carrier

protein synthases. β–keto-acyl-acyl carrier protein synthases are important for Type II fatty acid synthesis and are the main targets of antimicrobials: thiolactomycin, and cerulenin (Price *et al.*, 2001). Triclosan also inhibits fatty acid synthesis but had historically been assumed to have a mode of action related to cell membrane disruption, limiting bacteria nutrient uptake and growth (Heath *et al.*, 1998).

Fatty acids are a primary component of the phospholipid membrane. Phospholipid synthesis is an important biosynthetic pathway in all cells. Type II fatty acid synthesis is essential in several pathogenic bacteria (Yao and Rock, 2017; Young *et al.*, 2006). There are significant differences between the structures of the functional molecules involved in mammalian (Type I) and bacterial (Type II) which makes this pathway relevant and means mammalian cells may be safe from being targeted by these molecules (Yao and Rock, 2017; Young *et al.*, 2006).

3.5 Conclusions

Chitosans antimicrobial activity was studied emphasising how crucial characterisation and preparation details are in producing comparable results. Reporter strain exposure highlighted that different chitosans can induce different responses in gram positive *Bacillus subtilis*.

It is also worth noting that we did not screen chitosan against a reporter strain highlighting protein synthesis inhibition so chitosan may have targets there too. There are no research articles which compare chitosan activity to fatty acid synthesis inhibitors.

3.5.1 Further study

Carbohydrate microarrays of cell membrane components of different bacteria could help further identify differences in susceptibility between bacterial species to chitosan and further confirm and characterise whether there is binding between the membranes and chitosan.

Further understanding the way chitosan preparation impacts the antimicrobial activity will be beneficial for any future application of chitosan. A thorough framework for characterisation is also important to ensure chitosans meet the necessary standards in terms of quality and safety.

In addition to this it will be useful to further understand the interactions that chitosan may undertake with components of target foods. Anionically charged molecules may reduce chitosans efficacy as an antimicrobial.

3.5.2 Resistance

Raafat *et al.* (2017) investigated potential chitosan resistance mechanisms for *S. aureus*. They found that *S. aureus* was quick to develop resistance through modification of the cell wall. The modifications involved reduced overall negative charge. They urged caution with the indiscriminate use of chitosan as it may increase the occurrence of antimicrobial resistance. They observed changes in metabolism that may be related to the alterations of the cell wall structures. Therefore it is increasingly important that more research is directed towards understanding the mode of action and resistance mechanism chitosan may induce and the knock on effects this could have with increased chitosan applications.

3.5.3 Direction for food application

Chitosans effectiveness against several different bacteria highlight its broad spectrum but mild activity. From previous literature there is also some evidence that chitosan may induce antimicrobial resistance characteristics in some species which may increase consumer exposure risks in applying it to ready-to-eat food. Non-ready-to-eat food or food that needs high temperature treatment before consumption may be a good option for the application of chitosan in reducing degradative bacterial load.

3.6 References

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Chapter 4. Chitosan application as an edible coating

4.1 Introduction

4.1.1 Purpose of application

Following the different response of the *Bacillus* reporter strains to different chitosan samples and the risk previously highlighted in a research article towards inducing multi peptide resistance factor (Raafat *et al.*, 2017), it was decided that the primary focus of the application would be towards a non-ready-to-eat food. It was also decided that we would aim to reduce the spoilage rate of a highly perishable item.

4.1.2 Target food

Chitosan's research application as an edible coating is dominated by applications to fruits and vegetables. This is understandable due to the short shelf life of many of these foods. Fruits and vegetables are often spoiled by fungal species (Tournas and Katsoudas, 2005). For example *Botrytis cinerea* in strawberries (Tournas and Katsoudas, 2005) and other plant species. Chitosan's antifungal activity is species dependent in this group of organisms (Verlee, Mincke and Stevens, 2017). Its antibacterial efficacy is better evidenced in the literature and further highlighted in Chapter 3. In Chapter 3 we showed that chitosans inhibited gram positive and negative bacteria such as *E. coli*, *P. aeruginosa*, *B. subtilis*, and *S. cerro*. But chitosans did not inhibit the lactic acid bacterium *E. faecalis*.

It is also important to note that many of the issues related to food do not just arise from short shelf lives of foods. It includes foodborne diseases that are harmful to humans. It is easy to dismiss the pathogenic bacteria found on non-ready-to-eat foods, as most will be killed with thorough cooking. However, food poisoning still occurs on a frequent basis due to cross contamination when preparing foods. Therefore minimising pathogenic bacteria on raw meats is relevant.

4.1.3 Horizon scan

In order to better understand the food safety hazards most commonly associated with certain food groups. An assessment was undertaken using HorizonScan (2021), a platform which summarises and highlights global food hazard reports based on groups of foods and individual items. The most significant reported hazards to a food group were identified and

ranked by the top five reported hazards for each commodity group. Then the top five hazards for each item were ranked according to the antimicrobial characteristics that chitosan has shown in order to narrow down the target foods.

It was decided that there was a need to take into account the popularity of certain foods. For example rarely do people consume unspecified meat in the UK. The practicality of being able to acquire certain goods and the lost economic value that could potentially be prevented by applying a coating to further reduce the speed of deterioration.

4.1.4 Meat and poultry meat as a target food

Poultry are an important protein source with a low feed conversion ratio and can be significant for achieving the UN Sustainable Development Goals (FAO UN, 2022). It will play an important role in our future protein supplies. It is popularly consumed all around the globe. However the way it is raised and processed often lead to a risk of foodborne pathogens being present (Achen, Morishita and Ley, 1998). HorizonScan (2021) highlighted *Salmonella* species, *Campylobacter* species and *Listeria* species in the top five hazards for poultry meat (Table 23, 24a-c).

Salmonella occurs in the gut of animals and can cross contaminate raw meat, poultry, eggs and unpasteurised milk during processing (FSA, 2018b). *Salmonella* is the primary microorganism with which fresh poultry is tested for microbiological safety against EC 2073:2005 with presence in 25 g providing unsatisfactory results (EC, 2005). Salmonellosis is mostly problematic for young children and the elderly (FSA, 2018b). An EFSA study reported 80% of human salmonellosis cases are caused by *Salmonella enterica* and *Salmonella typhimurium* (EC, 2011).

Listeria is also problematic for young children, the elderly, the pregnant and the immunosuppressed (FSA, 2022). Cases are less common but *Listeria* is particularly associated with a wider range of foods including chilled ready-to-eat foods such as cured meats, pâté, smoked fish, and unpasteurised dairy (FSA, 2022).

Campylobacter are similarly more hazardous to young children and the elderly (FSA, 2018a). The primary source of *Campylobacter* infection in humans is through poultry products (Hermans *et al.*, 2011). *Campylobacter* is present in the intestinal tract of birds in high concentrations with *Campylobacter jejuni* responsible for most infections (Achen, Morishita

and Ley, 1998; Beery, Hugdahl and Doyle, 1988; Hermans *et al.*, 2011). Combining this with a low infective dose (FSA, 2018a) makes *Campylobacter* a public health concern in poultry products. It is also associated with gastroenteritis, and some other conditions (Gradel *et al.*, 2009).

The FSA generally advises the 4 C's for minimising microbiological hazards of foods which includes chilling below 8 ^{o}C, cooking thoroughly, avoiding cross contamination and cleaning surfaces and utensils thoroughly (FSA, 2018a). Due to poultry products being a main source of several foodborne pathogens (FSA, 2018a; FSA, 2018b; HorizonScan, 2021), raw meat must be handled with care and may increase the likelihood of food waste due to extra caution to organoleptic changes.

4.1.5 The framework of poultry meat processing

EC 2073 provides criteria for microbiological safety of foods including ready-to-eat and some raw characteristics (EC, 2005). EC 2073 draws upon the comments of the scientific committee on veterinary measures relating to public health highlighting raw meat and poultry as one of several food categories posing a high risk to public health in 2003.

EC 1086/2011 highlights an EU report evidencing *Salmonella* prevalence in broilers and turkeys as still high (EC, 2011). According to EC 2073, poultry meat may not be placed on the market for human consumption unless it meets the criterion: '*Salmonella* absence in 25 grams'. There is no further criteria for raw poultry meat other than mechanically separated meats.

Poultry legislation is more strict than other meats in terms of temperature and speed of processing after slaughter. EC 853:2004 states that carcasses must be cleaned and chilled to 4 ^{o}C as soon as possible and processed at no more than 4 ^{o}C for poultry, 7 ^{o}C for other meat, and within no more than three days of slaughter for poultry, whereas it is no more than six days for other animals (EC, 2004). For mechanically separated meat the poultry carcasses must be no more than three days old (EC, 2004). This highlights the high perishable and pathogenic risk to poultry.

4.1.6 Poultry meat decay

Meat spoilage is a product of microbial activity and metabolism altering the properties of the product to a degree at which the product is no longer accepted by the consumer due to unpleasant sensory characteristics (Bekhit *et al.*, 2021). Packaging systems, temperature, pH and the initial microbial composition present significantly contribute to the spoilage of meat (Kumar, Mukherjee and Dutta, 2020)

Raw poultry meat is highly perishable. Due to its high water activity, pH and nutritional composition (Bruckner *et al.*, 2012), it has ideal conditions for pathogenic and spoilage organisms (Latou *et al.*, 2014). The main criteria for poultry meat decay lies within altered organoleptic properties rather than by directly measurable qualities (FSA, 2010). Bruckner *et al.* (2012) compared the microbiological spoilage of porcine meat with poultry, concluding that microbial growth was faster on poultry than pork. The primary cause of poultry spoilage is associated with microbial growth (Bruckner *et al.*, 2012), in particular *Pseudomonas* species (Saenz-García *et al.*, 2020). Bruckner *et al.* (2012) showed that the growth of *Pseudomonas* species was directly related to temperature, with the increase in temperature increasing the rate of growth. Furthermore this highlighted the impact this can have on food waste and reinforced the reports regarding proper temperature storage.

Pseudomonas species are the main spoilage microbe on poultry meat during aerobic refrigeration (FSA, 2010). Aerobic colony counts (ACC)/Total viable counts (TVC) give an indication of the quality of a food product through an estimation of the microbiological load present. Approximately 7-8 log_{10}CFU g^{-1} is generally considered a maximum (Latou *et al.*, 2014; Wagle *et al.*, 2019) but chicken fillets can only be considered spoiled for consumption if organoleptic properties (taste, smell or appearance) are unacceptable or the meat is deemed unsafe with the presence of *Salmonella* in 25 g tested according to EC 2073 (EC, 2005). In ready-to-eat foods an ACC of 7-8 log_{10}CFU g^{-1} is considered a significant point in which gram negative bacteria, in particular *Pseudomonas*, begin to produce discolouration and slime in meat products (HPA, 2009).

4.1.7 Bacteria vs poultry processing

Poultry processing is responsible for the contamination of meat with hazardous microorganisms which usually occupy the intestinal tract of the birds (Achen, Morishita and Ley, 1998; Beery, Hugdahl and Doyle, 1988). In order to improve the safety of poultry meat,

processing plants introduce washing treatments to minimise the presence of bacteria on the surface of the meats. Peracetic acid has been developed to be used for decontaminating poultry carcasses, however bacteria such as *Campylobacter*, can survive this treatment and continue to cross contaminate during processing (Nagel *et al.*, 2013). The chemical is also an occupational risk to the safety of humans working in the vicinity (Pechacek *et al.*, 2015).

Chemicals such as chlorine, hydrogen peroxide and organic acid treatments have also been trialled, however they can have impacts on the organoleptic properties of the meat and their effectiveness as an antimicrobial treatment is limited (Northcutt *et al.*, 2005; Riedel *et al.*, 2009; Zhao and Doyle, 2006). Sinhamahapatra *et al.* (2004) compared four treatments on broiler carcasses using spraying and dipping. Hot water treatment at 70 ^{o}C, 2% lactic acid, 1200 ppm acidified sodium chlorite solution, and 50 ppm chlorine solution were the treatment formulations. They found that the hot water treatment and lactic acid treatments were slightly better at reducing the total viable counts compared with the untreated control, however the duration of testing only lasted for two days so inferences about the reduction in spoilage impacts could not be made.

Rather than washing treatments, antimicrobial coatings have seen more recent research attention due to their increased contact time with the meat surface due to retention of the coating and limiting the risk of contamination during handling due to the barrier created around the meat (Wagle *et al.*, 2019). These barriers can be designed to be selective to different properties and tailored towards the foods they are applied to. They can utilise different coating compositions and methods to enhance the properties of the barriers in different ways.

4.1.8 Coating Methods

There are various methods which can be used to apply a coating to a substrate. These include spraying, brushing and dipping (Khare *et al.*, 2016). There are also options for creating a film and applying the film as a wrap such as solvent casting, extrusion and electrospinning (Kumar, Mukherjee and Dutta, 2020).

Preparing films as a wrap for food can increase variability in the structure of the film and require equipment that is not always available on a laboratory scale. Extrusion is a useful

technique for plastic production and preferred over solvent casting due to its low energy usage and fast processing time (Kumar, Mukherjee and Dutta, 2020).

The variability introduced through preparing a film separately from applying it directly to the food also requires measuring the mechanical properties of the films using equipment which is not always accessible in different types of laboratories.

Solvent casting is one of the simplest forms of film preparation (Kumar, Mukherjee and Dutta, 2020) but is limited in scale up. Chitosan film formation was practiced in the lab and images taken (Figure 39). In order to solvent cast, the solution is spread on a surface (in this case a petri dish) and allowed to dry. It can then be peeled away from the base of the petri. This is useful for a visual representation of what is coating substrates in dipping procedures as they are not always visible, and permits testing of the mechanical and physicochemical properties of the composition.

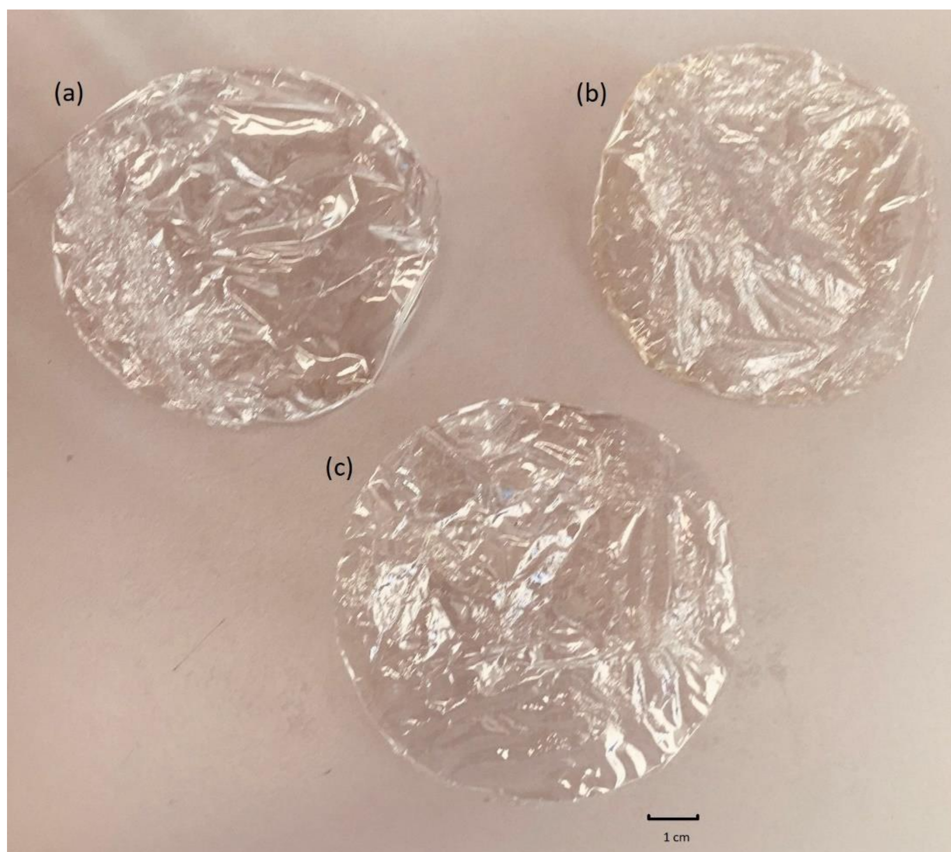

Figure 39 – Chitosan films 2% (w/v) in 1% (v/v) acetic acid formed by solvent casting method into a 90 mm petri dish with drying at ambient temperature (~ 20 ^{o}C) for 72 hours. (a) MMW chitosan (b) BSF chitosan (c) Squid chitosan with 1 cm scale bar.

Coating the substrate directly has been studied as a way of restricting the growth of microorganisms that may lead to an increase in shelf life and maintain organoleptic properties for longer. Khare *et al.* (2016) compared three different coating methods with a carrageenan based coating for chicken meat under refrigeration and found that they induced variation in the way they performed. Dipping and spraying induced a reduction in lipid oxidation in comparison to brushing, and dipping and brushing had similar TVC while spraying had a higher TVC. Sinhamahapatra *et al.* (2004) also compared poultry meat treatment methods by dipping and spraying in four different treatment formulations. They also found that the dipping methods were generally better at reducing the total viable counts than spraying. This highlights the variability in methods, possibly due to the quantity of coating remaining on the surface of the substrate.

Dipping has been a common method implemented in applying chitosan coatings to poultry meat in most studies. It is a simple technique which can be easily adapted to increase sample throughput and due to its simplicity can easily be performed. Dip composition also impacts the efficacy of the coating. Sagoo, Board and Roller (2002) examined two chitosan glutamate (42% glutamate, DDA 75-85%) dip concentrations effect against uncoated and pH adjusted water dipped samples on skinless pork sausages. They highlighted how important the concentration of the chitosan in the solution is as 0.5% chitosan had no difference in TVC compared with the pH adjusted control. Whereas a 1% chitosan coating had a measurable impact on reducing the TVC.

4.1.9 Chitosan dipping vs poultry meat studies

Maru *et al.* (2020) used a similar concentration of chitosan ('low' molecular weight, 75-85% DDA) to Sagoo, Board and Roller (2002). They used 1% (w/v) chitosan in 0.5% acetic acid with sterile distilled water as a control using the dip coating method. They highlighted a dramatic difference in bacterial counts between the control and chitosan treatment. Petrou *et al.* (2012) applied a slightly higher concentration of 1.5% (w/v) chitosan (MW '340' , DDA 75-85%) solution dissolved in 1% (w/v) acetic acid to chicken breast meat via the dip coating

method and monitored the properties of the meat over three weeks in modified atmosphere packaging. Both had poor background controls which did not highlight the impact that chitosan was having on the meat.

Olaimat and Holley (2015) also utilised modified atmosphere packaging with different coatings to assess the antimicrobial effect on *Salmonella* inoculated chicken breast meat. They evidenced that modified atmosphere packaging alone is not sufficient to prevent the growth of aerobic bacteria on chicken meat. They used an uncoated control with different chitosan (100-300 kDa, DDA 75-85%)/carrageenan coatings supplemented with further antimicrobials. Chitosan was dissolved at 2% (w/v) in 1% acetic acid with additional glycerol at 4% as a plasticiser. They too failed to highlight the impact that a similar pH solution to the chitosan would have on microbial growth. They did though show that chitosan in combination with vacuum packaging can reduce the growth of aerobic and lactic acid bacteria compared to uncoated. Latou *et al.* (2014) also examined the combination of chitosan coating with modified atmosphere packaging, applying a high molecular weight ((800,000) DDA 85%) 1% (w/v) chitosan in 1% acetic acid, but with aerobic and pH (1% acetic acid) controls. They highlighted a synergistic effect between the modified atmosphere and chitosan coating which in combination had the lowest TVC counts over the experimental period. However, modified atmosphere packaging may defeat the partial purpose of using biopolymers as coatings as typically they still involve the use of some single use plastics. Incorporating other methods such as enhancing the barrier properties or antimicrobial efficacy therefore have received research interest too.

Wagle *et al.* (2019) incorporated an antimicrobial extract of eugenol with their coatings of 2% (w/v) chitosan (Mw 190-310 kDa, no DA disclosed), in 50 mM acetic acid, with 50 mM acetic acid as a control with all adjusted to pH 6.5. They focussed on a pathogenic bacteria (*Campylobacter jejuni*) as well as the aerobic colony counts after application to chicken wingettes. They found that there was a synergy between the eugenol concentration and chitosan that had the lowest *C. jejuni* and aerobic colony counts after seven days. They concluded that this could be used as an effective postharvest treatment to reduce bacterial load on chicken wingettes. However, Wagle *et al.* (2019) did not assess the effect the potency of the extract may have had on the sensory properties of the food it is applied to.

Jafari *et al.* (2018) applied 2% (w/v) chitosan (mushroom derived, >75% DDA, ‘low’ molecular weight) coating in 1% acetic acid to chicken meat using a pH adjusted water solution as a

control dip. They also supplemented their coatings with propolis extract, a compound produced by bees, at different concentrations to see what effect that would have. They concluded that there were differences in the TVC counts between the control and chitosan throughout the 12 day testing period, with the control having a higher aerobic colony count throughout the experiment. The addition of the propolis also further reduced the colony counts throughout the experiment more so than the chitosan alone. Unlike Wagle *et al.* (2019), they utilised a sensory panel to examine the organoleptic properties of the treated meat. They found that the panel scored the control and chitosan similarly, however, those containing propolis extract were scored lower for odour and taste and therefore lower for overall acceptability.

These studies highlight the importance of using a good background control which accurately evidences the impact the acidic solution has on the ACC before the coating takes effect as days progress, and evidences that the chitosan concentration in the coating is appropriate to have an effect on the properties. It also highlights that there is limited further detail on the origin and characteristics of the chitosan utilised in these studies. To our knowledge no study has coated chicken meat in chitosan that has been characterised by both degree of acetylation (DA) and molar mass by light scattering/AF4. All previous studies have used chitosan in a similar DA range, none above 85% DDA, limited molar mass characterisation and rarely disclosed biological origin.

4.1.10 Layer-by-layer

Dipping can also facilitate the application of polyelectrolyte multilayers as a coating using oppositely charged polyelectrolytes in consecutive dipping solutions in a layer-by-layer (LBL) (Kumar, Mukherjee and Dutta, 2020) approach which may enhance barrier properties of the coating. LBL assembly has not been explored in as much rigour as single dip coatings. This LBL approach has mainly been applied to fruits and vegetables such as melon (Poverenov *et al.*, 2014b; Zhao *et al.*, 2020), bell pepper (Poverenov *et al.*, 2014a), strawberries (Yan *et al.*, 2019), cucumber and broccoli (Zhao *et al.*, 2020). But has also been applied to shrimp (Kim, Hong and Oh, 2018).

Poverenov *et al.* (2014a) applied chitosan and gelatin with brushing to bell peppers. With an uncoated control, gelatin and chitosan individually and combined, they measured the decay incidence of the pepper based on visible signs of microbial growth on the stem. The chitosan

alone had a greater reduction on the decay incidence. With gelatin providing the weaker reduction and the LBL coating providing a mid-range reduction. Poverenov *et al.* (2014b) applied chitosan and alginate to melon with different treatments including uncoated control, chitosan, alginate and combined LBL. Alginate had little effect, with similar TVCs to the uncoated control. Chitosan had the greatest TVC limitation and the LBL had slightly more growth than the chitosan.

Yan *et al.* (2019) applied chitosan and carboxymethylcellulose to strawberries. The control was dipped in distilled water. They did not examine microbial effects but found that firmness decreased more rapidly in control than other coatings. They reported that LBL delayed senescence during storage.

Zhao *et al.* (2020) applied chitosan and carboxymethyl chitosan with melatonin to broccoli, cucumber and melon. Antimicrobial susceptibility tests (AST) were performed but no TVC on the food. The AST revealed inhibition zones to *Listeria monocytogenes*, *Salmonella enterica* and *Escherichia coli*.

Kim, Hong and Oh (2018) applied chitosan and alginate to shrimp with uncoated controls. Coated shrimp showed significant differences in microbial counts by day 15 compared to the uncoated control. However, they did not shell the shrimp or remove the intestinal tract which may have introduced variability to counts. The treatments included uncoated, alginate, chitosan, combined in LBL in both configurations. Chitosan and LBL in which chitosan was applied first yielded similar but also the lower TVCs. With alginate and the LBL, where alginate was applied first, yielding similar TVCs but higher than the chitosan. The uncoated control had the highest counts quite considerably by day 15 but similar to the alginate at day 12.

4.1.11 Allergens

Insects have been highlighted to have a risk of contamination from microbiological hazards and have also been evidenced to accumulate heavy metals. Furthermore there is a risk of allergic reactions through varying routes including inhalant, skin contact, ingestion and stings and bites (De Marchi, Wangorsch and Zoccatelli, 2021). These include proteins such as tropomyosin, arginine kinase and myosin light chain.

Insects are taxonomically defined in the phylum Arthropoda which means they are related to several other known organisms which cause allergic reactions in humans including crustaceans and house dust mites. One protein called tropomyosin, associated with muscular contraction in arthropods, is partly responsible for allergic reactions in humans. 16 arthropod tropomyosins are registered as food allergens according to WHO/IUIS (De Marchi, Wangorsch and Zoccatelli, 2021), which share a common 3D alpha helical based structure. Black soldier fly does not currently have any registered allergens with WHO/IUIS and the allergens present are considered pan allergens of the Arthropod phylum.

Safety risks may pose a limitation to the application of insects. Therefore researchers have been looking at ways to minimise the risks in applying insects. These have included various processing methods to understand the impacts the methods have on the allergenicity of the processed material. van Broekhoven *et al.* (2016) highlighted how processing methods can impact the allergic potency of insects such as *Tenebrio molitor*, *Zophobas atratus* and *Alphitobius diaperinus*. They compared boiling, frying and freeze drying, and found that tropomyosin remained stable during boiling but that the allergenicity of the tropomyosin was reduced after frying.

Pali-Schöll *et al.* (2019) utilised enzyme and thermal processes on *Locusta migratoria* and found that both reduced the binding capacity of IgE. Leni *et al.* (2020) studied BSF allergenicity on crustacean allergic patients. They ground and froze the BSF and subjected the material to hydrolysis with a protease from *Bacillus licheniformis*. They found that the protein hydrolysate was still reactive towards IgE.

Verhoeckx *et al.* (2015) reviewed non-insect based allergen processing and concluded that processing will not abolish the allergenic potential of proteins. Microbial fermentation and enzymatic/acid hydrolysis may have potential to reduce allergenic integrity and allergenicity to prevent reactions being elicited.

4.1.12 Chitin/Chitosan allergenicity

Chitin is primarily derived from arthropods and molluscs for its use in various applications. The processing necessary to extract chitin and produce chitosan is considerable in comparison to the methods utilised for maintaining the proteins from insects for applications. Methods for determining chitin purity reliably are limited. Therefore it is often

assumed rather than guaranteed that extracted chitin and chitosan is free from the allergenic proteins present in insects.

There are not well documented reports of allergic reaction to chitosan, but contamination or incomplete purification is possible. This may mean that plant based alternative polymers are being used for applications instead of chitosan. However, chitosan is cationic and plant based polymers are mostly anionic. Multi polyelectrolyte based materials rely on the attraction of opposite charges. Therefore in creating multi polyelectrolyte based materials, chitosan is a fundamental biopolymer which can be used with alginate, pectin and other anionic polyelectrolytes.

4.1.13 Allergens methods

Allergens can be studied through direct and indirect methods. Polymerase chain reaction (PCR) is a molecular method employed for the confirmation of allergens in a sample and is useful for distinguishing the origin of an allergen (Eischeid, Stadig and Rallabhandi, 2021). PCR does not indicate whether the allergen is present or not, nor does it provide a guarantee that the allergen is still reactive. However unlike enzyme linked immunosorbent assay (ELISA), it is not affected by matrix interference and has a broad dynamic range, high levels of specificity, minimal cross reactivity and low limits of detection (Eischeid, Stadig and Rallabhandi, 2021).

ELISAs are commercially common methods of allergen assessments. They measure protein in a direct manner and provide an assessment of the reactivity of the allergen (Eischeid, Stadig and Rallabhandi, 2021). They can target total protein in a sample or a specific protein and there are several which are commercially available for tropomyosin for crustaceans. Due to the taxonomic relation between insects and crustaceans and evident cross reactivity of insect tropomyosin, the crustacean ELISA could be a viable detection method for confirming the absence/low level of insect tropomyosin in insect derived chitin and chitosan.

Allergen ELISAs are commonly performed in a sandwich format, can be performed without the need for advanced expertise or expensive lab equipment (Prado *et al.*, 2016). ELISA has several disadvantages compared to PCR including a narrow dynamic range which increases the minimum limit of detection and susceptibility to matrix interference which can induce false positives (Eischeid, Stadig and Rallabhandi, 2021; Prado *et al.*, 2016).

4.1.14 Aims and objectives

The aim of this chapter is to determine whether chitosan can improve the quality of foods in application as a coating material. Firstly sliced apple and diced fresh poultry meat were screened in initial assessments, this was then followed by application to fresh poultry meat.

Chitosan will primarily be tested against a pH control on raw chicken meat to see if it can limit deterioration over time. Total viable counts/aerobic colony counts will measure the bacterial growth, thiobarbituric acid reactive substances will assess the lipid oxidation of the meat and the pH of the meat will also be recorded to assess any significant deteriorations in quality. Chitosan will be applied in layer-by-layer assembly alongside food grade alginate to assess the effect of combining the polymers as a polyelectrolyte multilayer coating.

To our knowledge polyelectrolyte multilayers have not been assessed as a coating method for poultry meat. Chitosan/alginate multilayers have previously been examined but with limited benefit in comparison to chitosan application alone. Chitosan thoroughly characterised by its degree of acetylation and molar mass have also not been applied.

The **aims** and objectives for this chapter are further summarised:

4 Apply chitosan as a coating material to a food to reduce spoilage

4.1 Collect information regarding the perishable nature of foods using HorizonScan (2021)

4.2 Construct a narrative towards a specific food group utilising information gathered from HorizonScan (2021) and antimicrobial susceptibility tests

4.3 Measure the effects of different coatings on a food using total viable counts (TVC), thiobarbituric acid reactive substances (TBARS) and pH

4.4 Measure the effects of a chitosan compared to the control to determine if there are significant differences in TVC TBARS and pH values.

4.2 Materials and Methods

4.2.1 General Materials

4.2.1.1 Chemicals and consumables

Seward Filter Stomacher bags – Fisher Scientific UK

Spectrophotometer cuvettes - Fisher Scientific UK

Plate count agar - Fisher Scientific UK

Maximum recovery diluent 9 mL and powder - Fisher Scientific UK

1,1,3,3-Tetraethoxypropane >96% – Sigma Aldrich, Germany

Trichloroacetic acid 100% (w/v) Sigma Aldrich, Germany

2-thiobarbituric acid >98% - Sigma Aldrich, Germany

4.2.1.2 Equipment

Balance (0.01 g) Sartorius, Germany

UV spectrophotometer - Jenway, UK

Balances (0.1 mg) – Sartorius, Germany

Boiling water bath - Grant Instruments UK

SevenEasy pH meter - Mettler Toledo

Stuart Scientific colony counter

Interscience Laboratories Paddle blender

Eppendorf MiniSpin microcentrifuge

4.2.2 Visual assessment

Spoilage typically causes changes in sensory qualities in foods and so an initial visual assessment was undertaken with foods. Ice cube trays were sterilised by microwave for storage of dipped samples along with plastic containers. Cocktail sticks were placed in an oven at 200 ^{o}C for 10 minutes. Boiled water was used as a control and there was also an undipped control. Chitosan sample was prepared in acetic acid, 10cps chitosan at 3.5% (w/v)

in stoichiometric acetic acid. Diced raw chicken meat was purchased from a local supermarket along with gala apples. Gala apples were sliced on a chopping board wiped down with 10% bleach solution.

Chicken meat was dipped in the appropriate coating twice, allowed to dry for five minutes and then placed in the ice cube trays and sealed in the plastic containers. The plastic containers were then placed in the fridge at approximately 4 ^{o}C. There were two blanks (undipped), two water dipped controls and two chitosan dipped per apple chunk and per chicken meat.

The use by date stated on the chicken packaging was on the sixth day of the experiment. Images were taken on the sixth and 14th day after dipping. For the apples, the image was taken on the 25th day after dipping.

4.2.3 Allergen method

1 g of dried chitosan powder and powdered whole black soldier fly prepupae were transferred into separate 2 mL microcentrifuge tubes and secondly contained within falcon tubes. Placed into a plastic bag and posted for commercial testing with RomerLabs, UK through ELISA against the crustacean allergen tropomyosin.

4.2.4 Spoilage experimental setup

4.2.4.1 Round 1

Raw chicken mini fillets were purchased from a supermarket. Days 0, 4, 7, 11 were sampled. The use by date of the mini fillets indicated on the packaging landed on day 3 of testing.

4.2.4.2 Round 2

Similarly to the first stage of testing. Raw chicken mini fillets were purchased from a supermarket. Day 1, 4, 7, 10 were sampled. The use by date of the mini fillets indicated on the packaging was on day 3 of testing.

4.2.5 Coating preparation

4.2.5.1 Round 1

There were six different treatments investigated. The blank involved no treatment, the pH control was an acetic acid solution adjusted to pH 3.75 with NaOH, the alginate sample was food grade alginate previously characterised by ^{1}H NMR in Chapter 2, weighed and diluted to 10 g L^{-1} with a pH measured at 6.50, the 5cps was also characterised previously in Chapter 2 this was 10 g L^{-1} in stoichiometric acetic acid with a pH measuring at 3.75, the squid chitosan (SqCS) was previously characterised in Chapter 2 too, this was made up to 10 g L^{-1} with stoichiometric acetic acid with a pH of 3.80 and finally the layer-by-layer (LBL) coating utilised the 5cps, the alginate and the squid chitosan in successive dips. 1 mL of each coating solution was aliquoted and plated at each stage of the experiment for sterility. Once made the coating solutions were stored at 4 ^{o}C until use.

4.2.5.2 Round 2

There were two coating treatments involved in this experiment. The squid chitosan coating was at 10 g L^{-1} concentration in stoichiometric acetic acid with a pH of 3.79. The pH control involved a solution with the equal volume of acetic acid added to sterile distilled water and adjusted to pH 3.79 with NaOH. The coating samples were stored at 4 ^{o}C until use.

4.2.6 Coating application/setup

Aseptically using tweezers, Bunsen burner and 70% ethanol solution. Mini fillets were dipped into pots containing the coating three times and allowed to drip drain for 10 minutes before being placed into sterile Duran flasks, each flask containing the three mini fillets for a specific coating style and time point and replicate. The Duran flasks were then stored at 4 ^{o}C for the experiment until sampled. For round 2 there were three replicates per coating per time point, while in round 1 there was one replicate per coating per time point.

4.2.7 Total viable counts

At each time point, approximately 10 g was sampled from Duran flasks aseptically using scissors. This was then stomached in maximum recovery diluent (MRD) with a 1 in 10 dilution by weight for 60 seconds. The homogenate was then serially diluted in MRD and

pour plated 1 mL in standard 90 mm petri dishes with plate count agar (PCA). Dilutions were adapted as time progressed. Sterility controls were plated for coatings and agar itself. All dilutions were plated in duplicate. Plates were incubated at 30 ^{o}C for 72 hours. After 72 hours plates were removed from incubators, appropriate plates were identified with 30-300 colonies present and counted.

4.2.8 TBARS

4.2.8.1 Calibration

A calibration curve was set up for thiobarbituric acid reactive substances similarly to Maru *et al.* (2020) with minor modification. 75 mg of tetraethoxypropane (TEP) was diluted in 10 mL of 0.1 M HCl and placed into a boiling water bath then cooled. This was then diluted to 100 mL in water. This solution was then diluted in 1:4 in 10% trichloroacetic acid (TCA) and added to 0.02 M thiobarbituric acid (TBA) in a 1:1 ratio. This was then further diluted with water to produce a range to calibrate the quantity of malondialdehyde (MDA) through absorbance at 532 nm on a UV spectrophotometer.

4.2.8.2 Sample analysis

Thiobarbituric acid reactive substances was performed similarly to Maru *et al.* (2020) with minor modifications. On the appropriate time point, samples were macerated with a pestle and mortar and 2 g was weighed into a tube and 8 mL of 10% TCA was added. This was then agitated with a vortex mixer and centrifuged at 3000 x g for 10 minutes. 1 mL of the supernatant was added to 1 mL of 0.02 M TBA then heated at 95-100 ^{o}C in a boiling water bath for 30 minutes. After that the solution was transferred into a cuvette and absorbance measured at 532 nm on a UV spectrophotometer.

4.2.9 pH

On the appropriate time point, after samples were taken for microbial analysis, samples were macerated with a pestle and mortar and weighed out in a 1:5 dilution in sterile distilled water. The pH of the solution was then measured with a pH meter with agitation from a magnetic flea and stirrer at ambient temperature (~20 ^{o}C).

4.2.10 Analysis

IBM SPSS statistics Version 28.0.1.1 (15) was used for statistical analysis along with Microsoft Excel. Statistical analysis consisted of Kolmogorov-Smirnov normality tests, Levene's variance test and t-tests.

4.3 Results

4.3.1 Visual assessments

For the sliced apple chunks it was immediately visible that the chitosan coating had an impact on the spoilage of the apple. There were clear differences in the appearance of the top four apple chunks (controls) compared with the bottom two as illustrated in Figure 40a. The controls did not account for pH so further investigation would be necessary for evidently proving chitosan's activity and not the acidic nature of the coating.

Figure 40 – The different appearances of (a) sliced apple 25 days after dipping and raw diced chicken meat (b) six days after dipping (use by date) and (c) on the fourteenth day after dipping. The keys present on the images reflect the different treatments: i, ii. blank (no dip). iii, iv. deionised water dip. v, vi. chitosan dip. 10cps chitosan at 3.5% (w/v) in stoichiometric acetic acid.

As for the chicken meat there were also visible differences in the colouration of the meat when comparing before and after 14 days (Figure 40b, c). The darker colouration of the samples in wells c)i-iv was evident, while the chitosan coated samples remained a slightly paler colour associated with fresh chicken meat.

4.3.2 Allergen testing

The dried black soldier fly prepupae powder tested positive for crustacean tropomyosin within the quantification range with a value of 282.6 ppb. The dried chitosan powder isolated from black soldier fly did not have tropomyosin present at quantities measurable by ELISA and therefore fell below the limit of quantification for crustacean tropomyosin <20 ppb.

4.3.3 Round 1

4.3.3.1 Total viable counts

There were considerable differences in the total viable counts between differently treated poultry meat samples. At the beginning of the time series, day 0, it is clear from Figure 41 that the six samples fell into three different categories.

The blank poultry sample and alginate sample had high counts from the beginning with mean total viable counts of 7.34 log_{10}CFU g^{-1} and 7.21 log_{10}CFU g^{-1} respectively. These samples then reached the detection limits of the experiment as time progressed breaching the 8 log_{10}CFU g^{-1} mark by day 4 with mean total viable counts of 8.06 log_{10}CFU g^{-1} for the blank and 8.09 log_{10}CFU g^{-1} for the alginate.

The next pair of treatments with the middle range of initial counts were the pH control treated poultry meat and the lower molecular weight (5cps) chitosan treated meat. The pH control had a mean total viable count of 6.39 log_{10}CFU g^{-1} at day 0 while the 5cps chitosan had a mean total viable count of 6.24 log_{10}CFU g^{-1}. As time progressed, these two samples differed as the pH control count increased considerably more than the 5cps treated meat with means 7.45 log_{10}CFU g^{-1} and 6.40 log_{10}CFU g^{-1} at day 4 respectively. By day 7 the pH control reached the 8 log_{10}CFU g^{-1} mark, while the 5cps treated sample gradually increased to 7.72 log_{10}CFU g^{-1} at day 7. The 5cps treated meat reached the 8 log_{10}CFU g^{-1} mark on the final time point, day 11, with a mean total viable count of 8.70 log_{10}CFU g^{-1}.

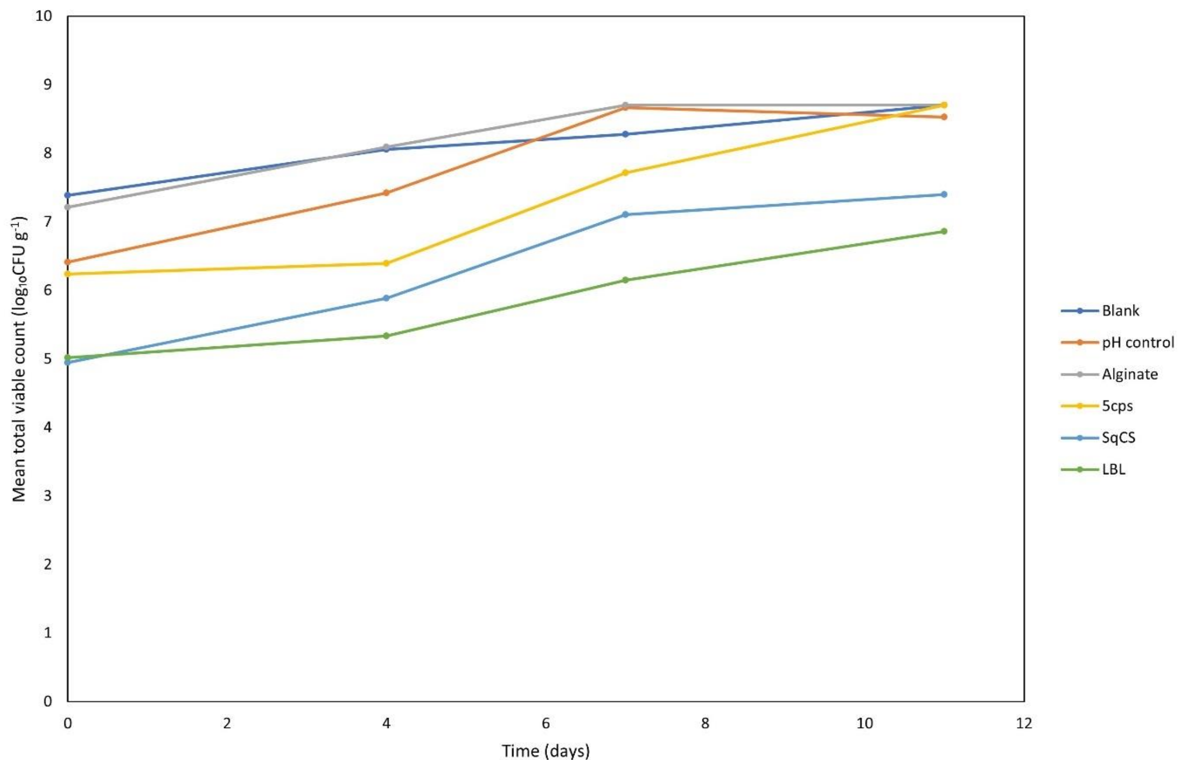

Figure 41 – Mean total viable counts of differently treated poultry meat samples over four time points (day 0, 4, 7, 11). Blank was undipped, pH control meat was dipped three times in an acidic sodium acetate solution at pH 3.75, alginate meat was dipped three times in 10 g L^{-1} food grade alginate at pH 6.50, 5cps meat was dipped three times in 10 g L^{-1} 5cps chitosan in acetic acid at pH 3.75, SqCS meat was dipped three times in 10 g L^{-1} squid derived chitosan in acetic acid at pH 3.80, Layer-by-layer (LBL) meat was dipped once in an aliquot of the 5cps solution, once in an aliquot of the alginate solution then once in an aliquot of the SqCS solution.

The final pair of treatments with the lower initial total viable counts were the higher viscosity chitosan (SqCS) and the meat samples treated with the layer-by-layer (LBL) assembly method which involved the 5cps, alginate and SqCS in sequential application. The SqCS treated poultry meat had an initial mean total viable count of 4.94 log_{10}CFU g^{-1} at day 0. The LBL treated samples had an initial mean total viable count of 5.01 log_{10}CFU g^{-1} at day 0. The mean total viable counts of both treatments increased for day 4 with 5.89 log_{10}CFU g^{-1} for SqCS and 5.44 log_{10}CFU g^{-1} for the LBL treated. A difference in counts begins to become visible in the figure at day 4 and then increased in size for day 7 where the SqCS had a

greater increase in total viable count than the LBL treated. The SqCS had a mean total viable count of 7.10 log_{10}CFU g^{-1} for day 7 and the LBL had 6.19 log_{10}CFU g^{-1}. By the final time point, day 11, neither treatments had counts above the 8 log_{10}CFU g^{-1} mark. The SqCS had a mean total viable count of 7.40 log_{10}CFU g^{-1}, while the LBL treated had the lowest mean total viable counts for day 4, 7 and 11 with a final mean total viable count of 6.86 log_{10}CFU g^{-1} for day 11.

4.3.3.2 Thiobarbituric acid reactive substances

4.3.3.2.1 Calibration

The calibration for the quantification of thiobarbituric acid reactive substances (TBARS) present in the poultry meat was performed through a series of dilutions of tetraethoxypropane (TEP) hydrolysed in mild hydrochloric acid. The absorbance and quantity of TEP were then inputted into SPSS, and a scatter plot was then generated (Figure 42).

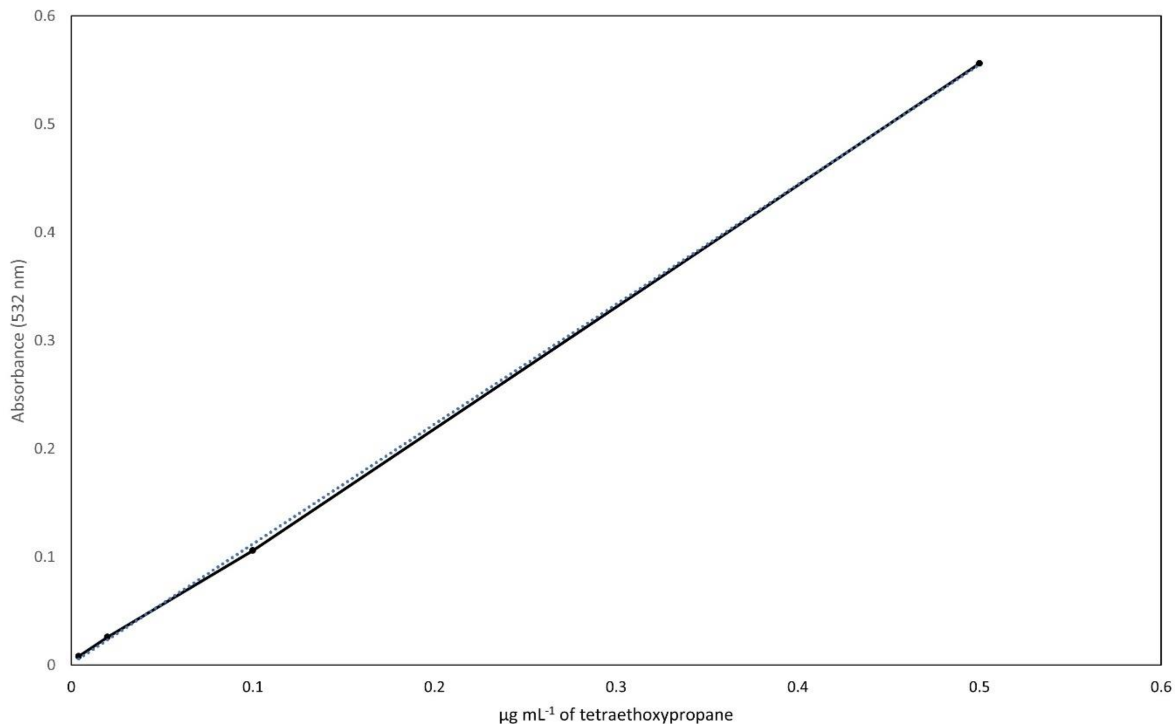

Figure 42- Calibration for thiobarbituric acid reactive substances using hydrolysed tetraethoxypropane. Y-axis = absorbance, x-axis = µg mL^{-1} of TEP. Line of best fit: y = 1.1073x + 0.0013. R^2 = 0.9998.

First normal distribution was tested using Kolmogorov-Smirnov. The TEP and absorbance values conformed to normal distribution (Kolmogorov-Smirnov, $P>0.05$) and a statistically significant positive correlation could be identified (Pearson Correlation Coefficient, $r = 0.9998$, $n = 4$, $P<0.001$). Regression analysis identified that absorbance at 532 nm significantly increased with increasing hydrolysed TEP used in the TBARS reaction (t-test, $t = 90.216$, $P<0.001$). The line of fit equation was $y = 1.1073x + 0.0013$, accounting for 99.98% of the variability in absorbance.

4.3.3.2.2 Sample analysis

TBARS has been used to measure the oxidation occurring in the treated meat samples. The different treatments had different patterns throughout the time points (days 0, 4, 7, 11).

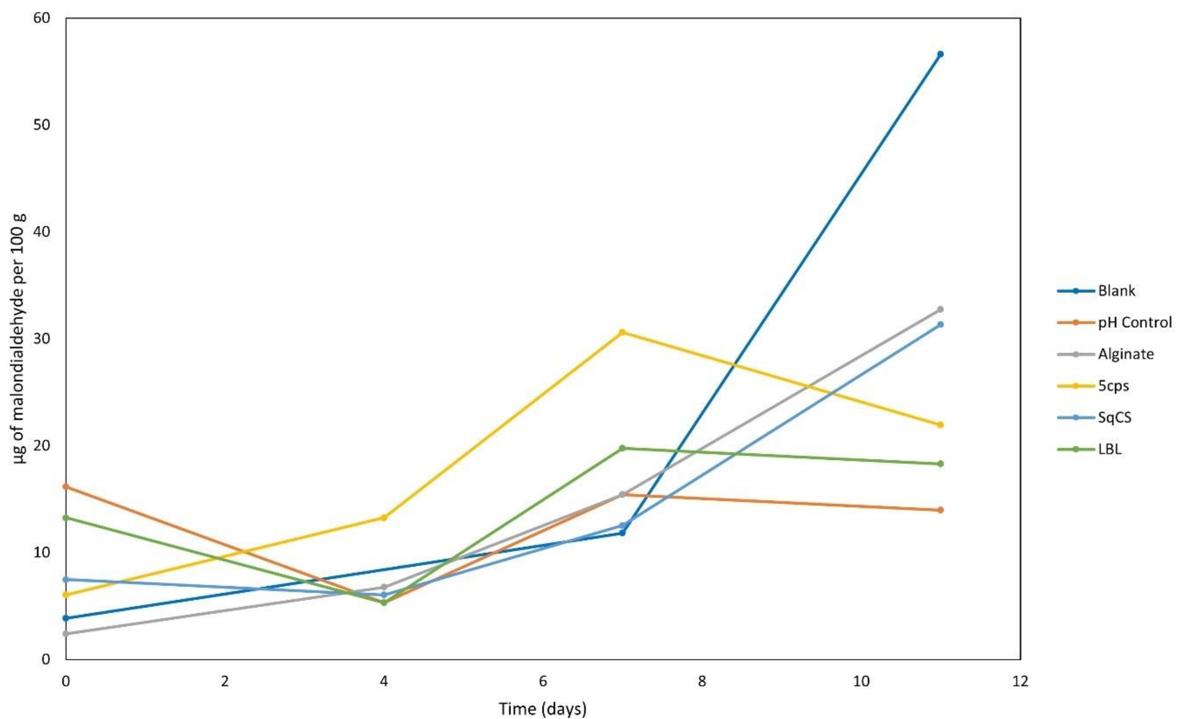

Figure 43 – Micrograms of malondialdehyde (TBARS) per 100 g of poultry meat over time with different surface treatments. Blank was undipped, pH control meat was dipped three times in an acidic sodium acetate solution at pH 3.75, alginate meat was dipped three times in 10 g L^{-1} food grade alginate at pH 6.50, 5cps meat was dipped three times in 10 g L^{-1} 5cps chitosan in acetic acid at pH 3.75, SqCS meat was dipped three times in 10 g L^{-1} squid derived chitosan in acetic acid at pH 3.80, Layer by layer (LBL) meat was dipped once in an aliquot of the 5cps solution, once in an aliquot of the alginate solution then once in an aliquot of the SqCS solution.

The different treatments shown in Figure 43, similarly to the patterns evident in the TVC analysis, could be paired in the initial quantities of TBARS measured. Firstly the blank and alginate treated samples showed the lowest initial values with 3.87 µg 100 g^{-1} and 2.42 µg 100 g^{-1} respectively. As time progressed the TBARS measured for these samples increased to provide the highest values by day 11 with the blank having 56.61 µg 100 g^{-1} and the alginate treated meat having 32.76 µg 100 g^{-1}. The blank had the highest TBARS reading on the final time point.

The second pair of treatments with the mid-range initial values of TBARS were the two treatments involving chitosans, 5cps and SqCS. They had TBARS values of 6.03 µg 100 g^{-1} and 7.48 µg 100 g^{-1} respectively at day 0. As time progressed the TBARS values for SqCS treated meat gradually increased to provide the third highest TBARS value at day 11 of 31.32 µg 100 g^{-1}.The 5cps treated meat had increased measured TBARS up to a peak on day 7 of 30.60 µg 100 g^{-1} but then slightly decreased for day 11 to 21.93 µg 100 g^{-1}, providing the fourth highest TBARS value for day 11 of the six treatments.

The final pair of treatments, the pH control and LBL treated meat, had the higher initial TBARS values on day 0 of 16.15 µg 100 g^{-1} and 13.26 µg 100 g^{-1} respectively. These samples had decreases in measured TBARS values for day 4, then a further increase at day 7 where it levelled off. By day 11 the TBARS values measured 13.98 µg 100 g^{-1} for pH control treated meat and 18.31 µg 100 g^{-1} for the LBL treated. This provided marginal differences in TBARS values between day 0 and day 11 for these treated meats and the lowest TBARS values of the six treatments.

4.3.3.3 pH

The pH values of the differently treated meats showed considerable differences between treatments and within treatments over the four time points (Figure 44). The blank (untreated) meat showed a gradual increase in pH from an initial pH of 5.8 at day 0 to 6.56 at day 11. The pH control showed a fairly stable pH which slightly increased with time with an initial pH of 5.93 on day 0 to 6.18 on day 11.

The alginate treated samples showed the greatest increase over time with an initial pH of 5.64 on day 0 to 7.23 on day 11. The alginate sample had the highest pH by quite a considerable margin by day 11. Of the five coatings used, the alginate coating was the sample with the highest pH at day 11.

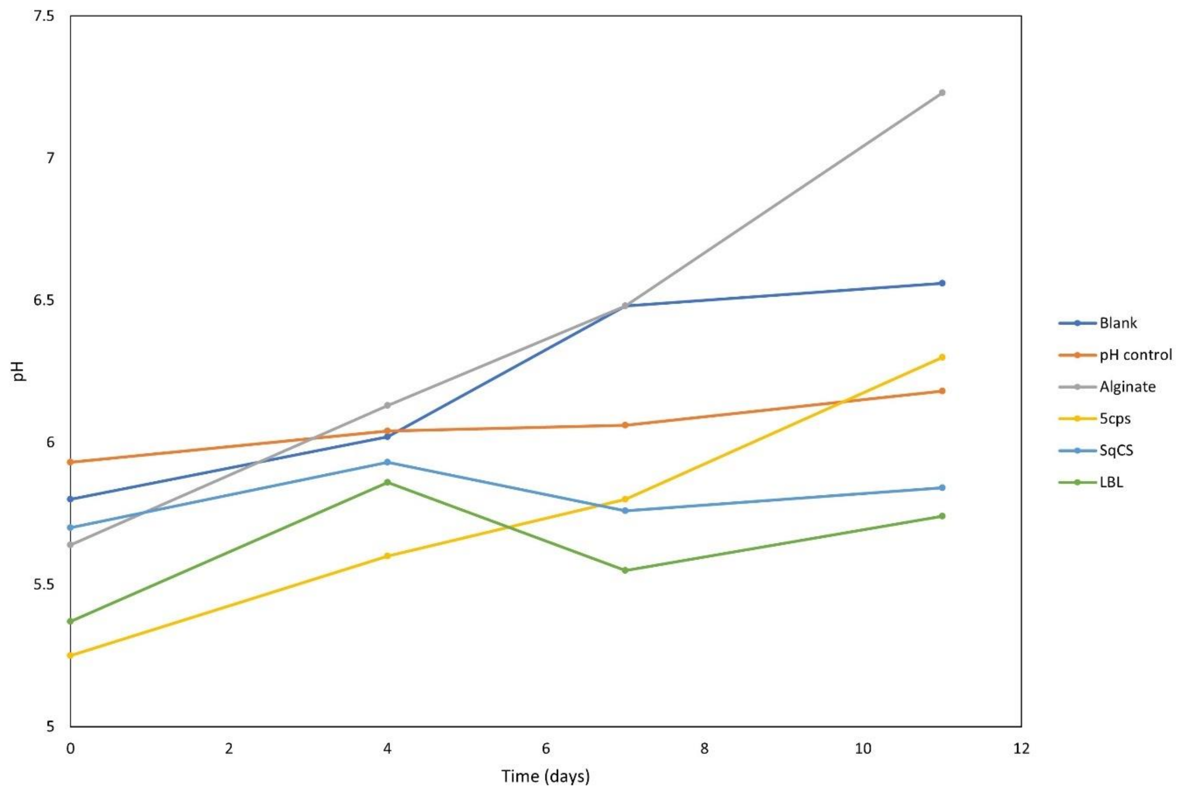

Figure 44 – pH measurements of homogenised poultry meat samples diluted in a 1:5 ratio with sterile distilled water over time with different surface treatments. Blank was undipped, pH control meat was dipped three times in an acidic sodium acetate solution at pH 3.75, alginate meat was dipped three times in 10 g L^{-1} food grade alginate at pH 6.50, 5cps meat was dipped three times in 10 g L^{-1} 5cps chitosan in acetic acid at pH 3.75, SqCS meat was dipped three times in 10 g L^{-1} squid derived chitosan in acetic acid at pH 3.80, Layer by layer (LBL) meat was dipped once in an aliquot of the 5cps solution, once in an aliquot of the alginate solution then once in an aliquot of the SqCS solution.

The 5cps treated poultry meat had the lowest initial pH at day 0 of 5.25 . The pH of 5cps treated meat gradually increased over time to a final pH of 6.3 on day 11. The higher viscosity chitosan sample, SqCS, had a pH pattern most similar to the pH control, showing some stability. The initial pH value at day 0 was 5.7, by day 11 the pH was 5.84. For the final treatment involving chitosan (LBL), the LBL treated meat showed minor pH fluctuations increasing from day 0 to day 4, decreasing from day 4 to day 7 then increasing again from day 7 to day 11. The initial pH of the LBL treated meat was 5.37 on day 0 while at day 11 it measured 5.74.

4.3.4 Round 2

4.3.4.1 Total viable counts

Mean total viable counts (log_{10}CFU g^{-1}) per gram of poultry meat were calculated from the plate counts at different dilutions and plotted against time to highlight the differences in microbial growth in raw poultry meat. For statistical analysis, normality tests were first performed using SPSS statistics.

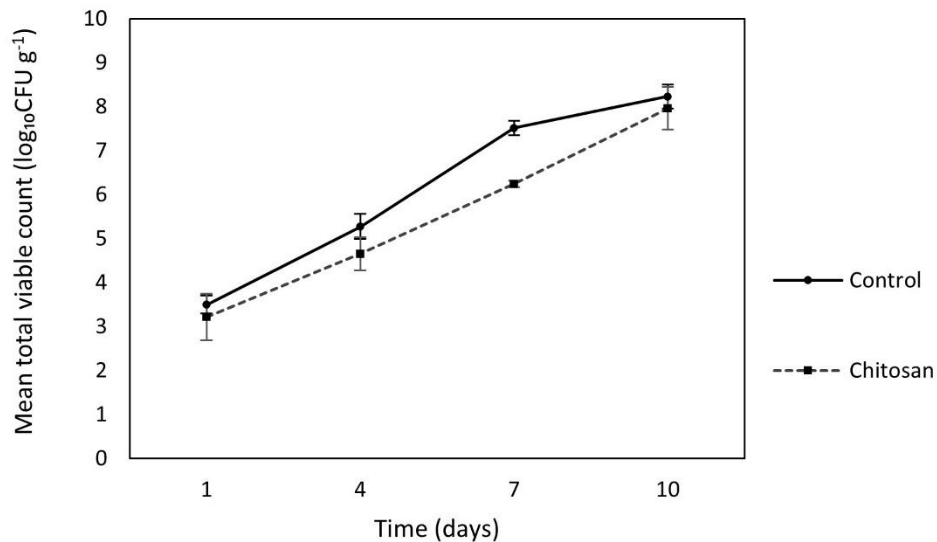

Figure 45 - Mean (± S.D.) total viable counts (log_{10}CFU g^{-1}) over time with different coating treatments. Control = pH acetate control pH 3.79, Chitosan = SqCS pH 3.79 1% (w/v).

4.3.4.1.1 Within sample

For the control, the values for each day conformed to normal distribution (Kolmogorov-Smirnov P<0.05). A series of t-tests were performed to confirm the positions illustrated on Figure 45 and summarised in Table 21. The variances of the data for day 1 and day 4 could be considered equal (Levene's test, F = 0.610 P>0.05) therefore the t-test assuming equal variances could be conducted. The t-test highlighted that the mean total viable count for day 4 (mean 5.28 ± 0.28 log_{10}CFU g^{-1}) was significantly higher than for day 1 (mean 3.50 ± 0.21 log_{10}CFU g^{-1}) (t-test, t = -12.402, df = 10 P<0.05). The variances of the data for day 4 and day 7 could be considered equal (Levene's test, F = 1.654 P>0.05) therefore the t-test assuming equal variances could be conducted. The t-test highlighted that the mean total viable count for day 7 (mean 7.52 ± 0.17 log_{10}CFU g^{-1}) was significantly higher than for day 4 (mean 5.28 ± 0.28 log_{10}CFU g^{-1}) (t-test, t = -16.603, df = 10 P<0.05). The variances of the data for day 7 and day 10 could be considered equal (Levene's test, F = 2.375 P>0.05) therefore the t-test assuming equal variances could be conducted. The t-test highlighted that the mean total viable count for day 10 (mean 8.23 ± 0.28 log_{10}CFU g^{-1}) was significantly higher than for day 7 (mean 7.52 ± 0.17 log_{10}CFU g^{-1}) (t-test, t = -5.378, df = 10 P<0.05).

Table 21- The outcomes of the Levene's variance test and t-tests performed when comparing the total viable count values over time for individual samples of background control dipped and chitosan dipped poultry meat. Statistical tests were performed on IBM SPSS statistics Version 28.0.1.1 (15). Y – Yes, N – No. Sign. diff. – significant differences as determined from the P value.

		Equal variance assumed	Sign. diff.
Control	Day 1 v day 4	Y	Y
	Day 4 v day 7	Y	Y
	Day 7 v day 10	Y	Y
Chitosan	Day 1 v day 4	Y	Y
	Day 4 v day 7	N	Y
	Day 7 v day 10	N	Y

For the chitosan treated sample, the data also conformed to normal distribution (Kolmogorov-Smirnov $P<0.05$). A series of t-tests were performed to confirm the positions illustrated on Figure 38. The variances of the data for day 1 and day 4 could be considered equal (Levene's test, $F = 1.200$ $P>0.05$) therefore the t-test assuming equal variances could be conducted. The t-test highlighted that the mean total viable count for day 4 (mean 4.66 ± 0.38 $log_{10}CFU\ g^{-1}$) was significantly higher than for day 1 (mean 3.22 ± 0.53 $log_{10}CFU\ g^{-1}$) (t-test, $t = -5.427$, $df = 10$ $P<0.05$). The variances of the data for day 4 and day 7 could not be considered equal (Levene's test, $F = 23.283$ $P<0.05$) therefore the t-test not assuming equal variances could be conducted. The t-test highlighted that the mean total viable count for day 7 (mean 6.24 ± 0.07 $log_{10}CFU\ g^{-1}$) was significantly higher than for day 4 (mean 4.66 ± 0.38 $log_{10}CFU\ g^{-1}$) (t-test, $t = -10.102$, $df = 5.346$ $P<0.05$). The variances of the data for day 7 and day 10 could not be considered equal (Levene's test, $F = 7.786$ $P<0.05$) therefore the t-test not assuming equal variances could be conducted. The t-test highlighted that the mean total viable count for day 10 (mean 7.96 ± 0.49 $log_{10}CFU\ g^{-1}$) was significantly higher than for day 7 (mean 6.24 ± 0.07 $log_{10}CFU\ g^{-1}$) (t-test, $t = -8.570$, $df = 5.210$ $P<0.05$).

4.3.4.1.2 Between samples

For the samples measured on day 1, 4, 7 and 10 normality tests concluded that both the chitosan treated and control total viable counts conformed to normal distribution (Kolmogorov-Smirnov, $P>0.05$), therefore t-tests were conducted. Before proceeding with

the t-tests, the testing for equal variances was conducted. A summary of the tests performed and outcomes are provided in Table 22.

Table 22 – The outcomes of the Levene's variance test and t-tests performed when comparing the total viable count values between background control dipped and chitosan dipped poultry meat samples. Statistical tests were performed on IBM SPSS statistics Version 28.0.1.1 (15). Y – Yes, N – No. Sign. diff.-significant differences as determined from the P value.

	Equal variance assumed	Sign. diff.
Day 1	N	N
Day 4	Y	Y
Day 7	N	Y
Day 10	Y	N

For day 1 equal variances could not be assumed ($F = 7.882$, $P<0.05$) therefore a t-test with equal variances unassumed was conducted. The t-test concluded that there was no statistically significant differences in the total viable counts for chitosan treated (mean 3.22 ± 0.53 $log_{10}CFU\ g^{-1}$) and the control treated (mean 3.50 ± 0.21 $log_{10}CFU\ g^{-1}$) poultry meat for day 1 (t-test, $t = 1.212$, $df = 6.504$, $P>0.05$).

The day 4 samples of chitosan treated and control treated could be considered to have equal variances (Levene's test, $F = 1.607$, $P>0.05$). However, with equal variances assumed the t-test highlighted a statistically significant difference between the chitosan treated (mean 4.66 ± 0.38 $log_{10}CFU\ g^{-1}$) and control treated (mean 5.28 ± 0.28 $log_{10}CFU\ g^{-1}$) poultry meat total viable counts (t-test, $t = 3.207$, $df = 10$, $P<0.05$).

For the samples measured on day 7 the variances of the chitosan and control treated poultry meat could not be considered equal (Levene's test, $F = 6.002$, $P<0.05$). With equal variance unassumed, the t-test then indicated that there was a statistically significant difference between the mean total viable counts of the chitosan treated (mean 6.24 ± 0.07 $log_{10}CFU\ g^{-1}$) and control treated (mean 7.52 ± 0.17 $log_{10}CFU\ g^{-1}$) poultry meat (t-test, $t = 16.937$, $df = 6.674$, $P<0.05$).

Finally total viable counts from the chitosan treated and control treated poultry meat on day 10 could be assumed to have equal variances (Levene's test, $F = 1.326$, $P>0.05$). The t-test

assuming equal variances highlighted no statistically significant differences between the total viable counts of chitosan treated (mean 7.96 ± 0.49 log_{10}CFU g^{-1}) and control treated (mean 8.23 ± 0.28 log_{10}CFU g^{-1}) poultry meat (t-test, t = 1.171, df = 7.945, P>0.05).

4.3.4.2 TBARS

4.3.4.2.1 Within sample

The control treated and chitosan treated meat had similar TBARS results (Figure 46). The control treated sample increased initially up to day 7 but then began to show a decrease for the final time point at day 10. The chitosan treated sample also increased initially but then decreased slightly after day 4 before increasing again after day 7.

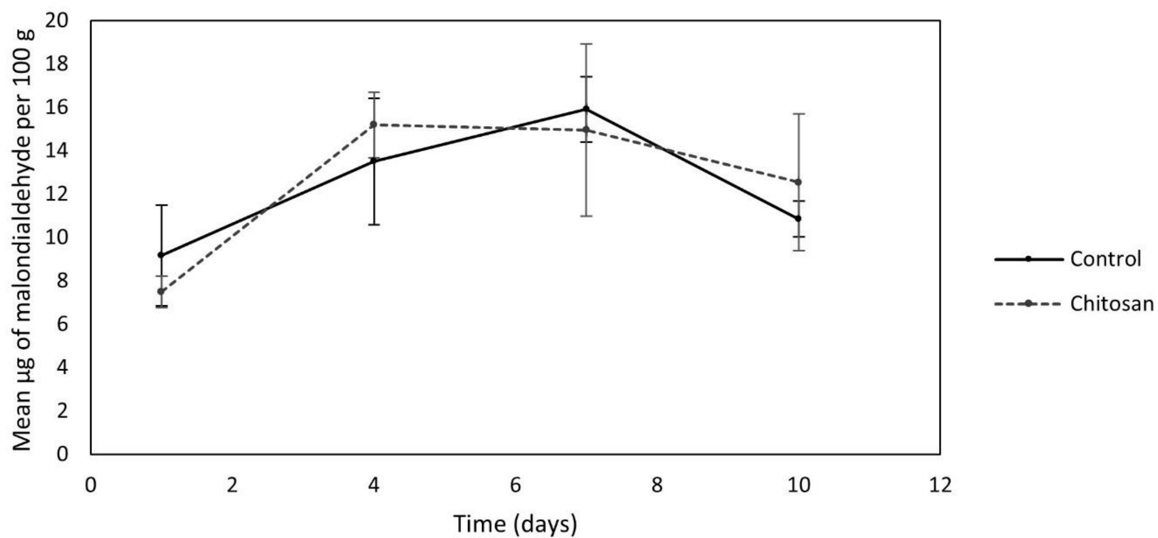

Figure 46 – Mean (± S.D.) micrograms of malondialdehyde per 100 g of chicken meat determined by thiobarbituric acid reactive substances with different coating treatments over time (pH control pH 3.79, SqCS pH 3.79 1% (w/v) CS)

4.3.4.2.2 Between samples

Both samples showed good precision with low variability for the initial measurements on day 1, the control measured mean 9.16 ± 2.32 µg 100 g^{-1} and the chitosan treated measured mean 7.48 ± 0.72 µg 100 g^{-1}. Both samples increased similarly up to day 4, control treated meat showed poor precision with a high S.D. (mean 13.50 ± 2.92 µg 100 g^{-1}). Chitosan

treated measurements at day 4 showed good precision with a low S.D. (mean 15.18 ± 1.50 µg 100 g^{-1}). For day 7 measurements, chitosan treated meat showed higher variability between measurements while the control treated showed higher precision. Chitosan treated had a mean TBARS quantity of 14.94 ± 3.98 µg 100 g^{-1} while control treated had a mean TBARS quantity of 15.91 ± 1.50 µg 100 g^{-1}. For the final measurements on day 10, the control treated had a decrease from day 7 with a mean TBARS quantity of 10.85 ± 0.83 µg 100 g^{-1} while the chitosan treated had a minor increase from day 7 with a mean TBARS quantity of 12.53 ± 3.15 µg 100 g^{-1}.

4.3.4.3 pH

The pH measurements highlighted similarities in the control and chitosan treated poultry meat (Figure 47). The pH changes throughout the time points remained within a 0.6 pH range. The control treated showed good pH stability. At day 1 the pH had a mean of 6.3 ± 0.01 which remained similar by day 4 with a mean of 6.32 ± 0.06 .There was a slight decrease in the mean by day 7 which measured at 6.22 ± 0.04. At day 10 the pH remained similar to day 7 with slightly more variability with a mean of 6.21 ± 0.09 .

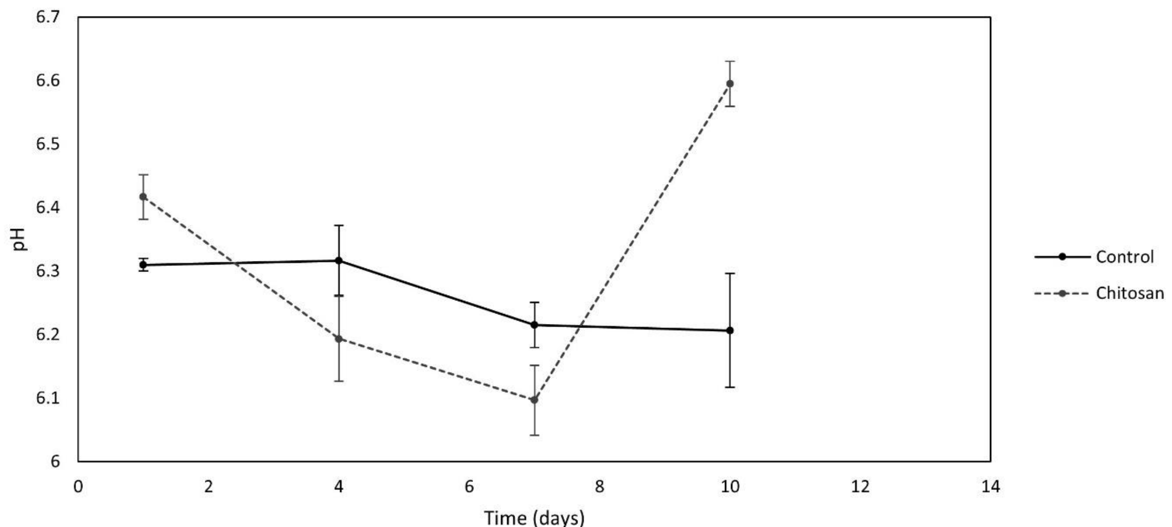

Figure 47 - Mean (± S.D.) pH measurements for control treated and chitosan treated raw poultry meat over time. pH acetate control pH 3.79, SqCS pH 3.79 1% (w/v) CS.

The chitosan treated showed slightly poorer pH stability than the control treated poultry meat. At day 1 the meat had a mean pH of 6.42 ± 0.04 S.D. The pH decreased to day 4 (mean 6.19 ± 0.07 S.D.) and down to day 7 (mean 6.10 ± 0.06 S.D.) before increasing quite significantly by day 10 measuring a mean pH of 6.60 ± 0.04 S.D.

4.4 Discussion

4.4.1 TVC

4.4.1.1 Comparisons between experiments

The samples used for both shelf life experiments were similar and comparable as replicates. The pH control and the SqCS samples used similar formulations. Both experiments used chicken meat with use by dates stated on the packaging that occurred at similar stages of the experiment.

The samples in the second experiment had considerably lower initial TVCs than the samples in the first experiment. The first experiment therefore highlighted the initial antimicrobial impact the coatings had on the chicken meat. This was broken down into three differently performing coating sets with two coatings in each. The blank and alginate coatings performed similarly, with TVCs exceeding 7 $log_{10}CFU\ g^{-1}$ from day 0. The pH control and 5cps samples were mid-range in reducing microbial loads with approximately 6 $log_{10}CFU\ g^{-1}$. While the LBL and SqCS having the greater initial impact on microbial load with approximately 5 $log_{10}CFU\ g^{-1}$. However in the second experiment, the difference in the initial TVCs between pH control and SqCS were less prominent with both measuring at approximately 3 $log_{10}CFU\ g^{-1}$.

As time progressed, microbial growth varied among differently coated samples. In experiment two, the pH control TVC increased more rapidly than the chitosan treated, leading to significantly higher TVCs for the pH control at day 4 and day 7. The pH control sample reached the 8 $log_{10}CFU\ g^{-1}$ by the end of the experiment, similarly to the pH control in experiment one. The SqCS sample also had a lower TVC than the pH control throughout experiment one. With the SqCS remaining below 8 $log_{10}CFU\ g^{-1}$. While the LBL coating outperformed the chitosan alone with slower microbial growth with a final value below 7 $log_{10}CFU\ g^{-1}$.

The different chitosan coatings which were applied at the same concentration and same pH in experiment one highlighted that the chitosan characteristics may have an impact on the coating performance. This is most likely due to the increased viscosity of the higher molecular weight SqCS coating which will have meant more coating was retained on the sample during dipping but could also be related to the degree of acetylation.

4.4.1.2 Comparisons with the literature

The chicken meat used in experiment two was of a comparable initial microbial load to several other studies (Jafari *et al.*, 2018; Latou *et al.*, 2014; Petrou *et al.*, 2012; Wagle *et al.*, 2019). Petrou *et al.* (2012) measured an initial 4.85 $log_{10}CFU\ g^{-1}$ for fresh chicken meat similarly to Latou *et al.* (2014) who measured 4.1 $log_{10}CFU\ g^{-1}$ initially, Jafari *et al.* (2018) at approximately 4 $log_{10}CFU\ g^{-1}$ and Wagle *et al.* (2019) had an initial TVC of approximately 4.5 $log_{10}CFU\ g^{-1}$. However, Maru *et al.* (2020) reported 7.27 $log_{10}CFU\ g^{-1}$ for day 0 of their experiment. This highlights the variability in the microbial composition of fresh poultry meat and suggests that the meat used in experiment one and in Maru *et al.* (2020) may have been further along in its spoilage than the other studies when the experiment began. It may also have been from different origins and batches.

Jafari *et al.* (2018) noted an increase in TVC from 4 $log_{10}CFU\ g^{-1}$ to 8.1 $log_{10}CFU\ g^{-1}$ over 12 days in aerobic conditions for a control dip while the chitosan treated had a reduced TVC of 7.4 $log_{10}CFU\ g^{-1}$. Wagle *et al.* (2019) measured an increase in TVC for their control from approximately 4.5 to 6 $log_{10}CFU\ g^{-1}$ over seven days. Meanwhile Wagle *et al.* (2019) observed an initial reduction in the TVC for the chitosan treated compared to the control and observed an increase in TVC from 3 to 5 $log_{10}CFU\ g^{-1}$ over the seven days. These results are comparable to experiment two in which we observed increased TVCs for the control over the chitosan that lead to statistical significance on day 4 and day 7 but eventually the chitosan treated meat TVC increased at day 10 with a similar value to the control (approximately 8 $log_{10}CFU\ g^{-1}$).

4.4.1.3 LBL antimicrobial synergy

Composite materials have gained a significant amount of research attention due to the synergistic effects of multiple compounds. Wagle *et al.* (2019) and Jafari *et al.* (2018) introduced additional antimicrobials to reduce microbial load further. Wagle *et al.* (2019) introduced eugenol to chitosan and pectin coatings, which appeared to slow the increase in TVC over the seven day testing period compared with chitosan, the greatest concentration of eugenol combined with the chitosan had the greatest effect, with a final TVC at day 7 of approximately 4.5 $log_{10}CFU\ g^{-1}$. The LBL coating in experiment one allowed an increase in TVC of approximately 1.5 $log_{10}CFU\ g^{-1}$ which is comparable to this but the eugenol is more likely to have an impact on sensory characteristics like taste and smell. Similarly to Wagle *et*

al. (2019), Jafari *et al.* (2018) used propolis extract concentrations and showed that with a 2% concentration added to the chitosan coating, it could reduce the microbial growth by approximately 1 log_{10}CFU g^{-1} over 12 days. This is also similar to the LBL coating applied in experiment one in which the coating appeared to suppress the increase in TVC compared to the chitosan coatings alone.

The LBL has not previously been applied to meat products and the effects of chitosans with different characteristics have not been compared. But Poverenov *et al.* (2014b) applied chitosan-alginate LBL to melon. Contrary to what was observed in experiment one, Poverenov *et al.* (2014b) observed that chitosan alone was better at limiting the increase in TVC than the LBL coating.

Chitosan-alginate layers have also been applied to shrimp (Kim, Hong and Oh, 2018). Kim, Hong and Oh (2018) found that chitosan and LBL chitosan-alginate coatings incorporated with grapefruit seed extract, in which chitosan is first applied to the substrate, did not vary significantly in their ability to reduce TVC on shrimp. The coatings were comparatively better than uncoated controls and when alginate is applied to the substrate first in LBL technique. Kim, Hong and Oh (2018) however, did not use a background control for the impact the pH and the grapefruit seed extract may have had on the TVC reduction, and did not remove the shell or intestinal tract from the shrimp which may had implications for the TVC and the ability for the coating to have a penetrating antimicrobial effect further.

4.4.2 TBARS

4.4.2.1 Comparisons between experiments

The pH controls were also comparable, as in experiment one the value at day 0 was 16.15 μg $100g^{-1}$ while in experiment two the value at day 1 was 9.16 μg $100g^{-1}$, indicating that there was some variation in oxidation at the beginning of the experiments. As time progressed the pH control in experiment one remained at a similar TBARS value of 13.98 μg $100g^{-1}$ at day 11, while the pH control in experiment two also declined from its mid-experiment peak with a final value of 10.85 μg $100g^{-1}$ at day 10.

At the start of both experiments, the TBARS values for SqCS were comparable as experiment one measured 7.48 μg $100g^{-1}$ on day 0 and in experiment two measured 9.16 μg $100g^{-1}$ on day 1. However as time progressed the TBARS values measured were considerably different.

SqCS gradually increased in TBARS value to 31.32 µg $100g^{-1}$ at day 11 in experiment one, while in experiment two the SqCS reached 12.53 µg $100g^{-1}$ by day 10.

4.4.2.2 Comparisons with the literature

Oxidative rancidity is understood to become noticeable in meats in the range of 100-200 µg MDA 100 g^{-1} (Latou *et al.*, 2014; Maru *et al.*, 2020; Paparella *et al.*, 2016; Vaithiyanathan *et al.*, 2011). In both experiments our values did not exceed the lower bound (100 µg 100 g^{-1}) value of the threshold, indicating that lipid oxidation was not a major cause of meat spoilage in these cases.

The maximum value reached in our experiments was achieved with the uncoated sample in experiment one which yielded a TBARS value of 56.61 µg 100 g^{-1} on day 11. This was followed by the alginate sample with a value of 32.76 µg 100 g^{-1} at day 11. The pH values of these samples were also the highest two on day 11 of this experiment indicating a possible link.

These values are similar to Petrou *et al.* (2012) who reported low levels of lipid oxidation which did not exceed 50 µg 100 g^{-1}. Latou *et al.* (2014) also had comparable values beneath the threshold for oxidative rancidity, reporting values in the range of 19 to 80 µg 100 g^{-1}.

Maru *et al.* (2020), however, noted considerable oxidative rancidity of chicken meat, but the duration of their experiment was 16 days as compared to the 11 we performed. It would be useful to continue the experiment for longer to determine whether oxidation increases more over time as they reported. On the other hand, Petrou *et al.* (2012) and Latou *et al.* (2014) both highlighted that modified atmosphere packaging was sufficient to deter oxidative rancidity with a study duration of 21 days and 14 days respectively. Maru *et al.* (2020)'s control of sterile distilled water dipping produced a value of 142.7 µg 100 g^{-1} on day 16, while chitosan treated meat reached 90.3 µg 100 g^{-1} by day 16. It is generally considered that chitosan has antioxidative properties and so this measured reduction for Maru *et al.* (2020) was as expected. However with Maru *et al.* (2020) not using a pH control dip, and our own results which show that the pH control yielded similar TBARS values to the chitosan treated, it remains unclear whether chitosan is solely responsible for this effect.

4.4.3 pH

4.4.3.1 Comparisons between experiments

Between the two spoilage experiments performed there were considerable differences in the pH values obtained as the experiment progressed. The pH control had a stable pH throughout experiment one ranging from 5.93 to 6.18. The pH control in experiment two also remained stable but with a slightly higher pH than experiment one, with values as expected for fresh chicken meat, ranging from 6.32 to 6.21.

The SqCS sample in experiment one also showed a fairly stable pH with values ranging between 5.70 and 5.84. While the SqCS used in experiment two had a wider ranging pH throughout experimentation, from 6.10 to 6.60. There are considerably different pH values of the meat between experiments indicating differences in conditions possibly due to oxidation or microbial spoilage. The blank uncoated sample showed a steady increase in pH as time progressed, while the alginate sample showed the greatest range, increasing from 5.64 to 7.23 over the 11 days.

4.4.3.2 Comparisons with the literature

It is generally agreed in the literature that fresh chicken meat has a pH of approximately 6.2 (Latou *et al.*, 2014; Maru *et al.*, 2020; Petrou *et al.*, 2012). Maru *et al.* (2020) observed a significant drop in pH value for a control sample over the 16 days of their experiment in which the control sample reached 5.6 pH. However, Latou *et al.* (2014) noted an early acidic pH of 5.85 and attributed this to the pH of the coating solutions. While Khare *et al.* (2016) did not use chitosan, but did use acidic coatings, they also highlighted that lower values of pH could be due to the coating pH. In experiment one we utilised six different coatings, four chitosan (acidic) coatings, one undipped and one sample at pH 6.5. But did not observe any obvious separations in pH values at the initial sampling point that could be conclusively associated with the pH of the coating solution. However, it was evident that the samples that were dipped in one particular coating containing low viscosity chitosan, which included both the 5cps and LBL coating treatments, did produce the slightly lower pH values. This indicates there may be other factors contributing. For example the chicken meat could be at different stages of its spoilage. Also, the microbial compositions present on the chicken could be different, contributing to differences in conditions. Degradation processes such as oxidation and protein degradation could be at different stages. The retention of the coating

on the surface of the meat, as well as the molecular size of the coating components could contribute to effects relating to the coatings ability to penetrate into the meat too.

Petrou *et al.* (2012) had stable pH values for all coating treatments, falling in the range of 6.1 to 6.3, with no statistically significant differences between them. In experiment two, the pH control sample showed comparable behaviour to this study, with pH values falling between 6.32 and 6.21 throughout experimentation. In experiment two, the pH of the chitosan treated sample followed a similar pattern to the pH control sample. But on the final day (11) the pH increased quite dramatically creating a larger range between 6.10 and 6.60. This could be indicative of the quantity of the coating being sampled impacting the pH due to the technical challenges of coating evenly and sampling the coating in equal quantities.

4.4.4 Allergens

The commercial crustacean ELISA for the powdered black soldier fly prepupae quantified 282.6 ppb of tropomyosin present. While ELISA for the chitosan powder isolated from the black soldier fly prepupae was unable to quantify a value as it was below the limit of quantification of 20 ppb. This indicates that the chemical processing of insect material to chitosan greatly reduces the quantity of tropomyosin present, but does not confirm that it is completely absent. Complete absence could be further determined through extra testing via more sensitive proteomics based methods such as the combination of liquid chromatography and mass spectrometry (Prado *et al.*, 2016). It has been previously evidenced that chitosan and chitin can contain residual immunogenic tropomyosin within the matrices (Nguyen, 2012).

4.5 Conclusions and future directions

The experiments performed in this chapter evidenced that the chitosan characteristics can impact the performance of a chitosan based coating. They evidenced that chitosan can statistically significantly impact the TVC on chicken meat at certain time points. Furthermore they highlighted that composite coatings have potential for further optimising the coating performance on specific foods. In particular layer-by-layer assembly, and nanocomposite methods hold great potential that need investigating further.

In experiment one the LBL coating appeared to have a synergistic effect at reducing the increasing TVC in chicken meat. However, there were limitations in the number of replicates performed and therefore further attention could be directed at increasing the replicates. As well as this, further attention could be directed to the order of assembly. While Kim, Hong and Oh (2018) highlighted that applying chitosan to the substrate first appeared more effective, there could be other combinations with other biopolymers, and additives that further enhance the coating performance, for example chitosan nanoparticles.

The use of alginate or pectins opens up opportunities to consider collaboration with sustainable seaweed farming or vertical farming which could provide further diverse compounds for trial in coatings. In addition reactive molecules which indicate changes in environmental conditions like pH could be developed into these coatings.

Furthermore, it is paramount that the overall purity of the compounds used in edible coatings are defined. The edible coating is a food component and therefore significant consideration must be taken for consumer safety, whether that be the migration risk of contaminants, the risk of inducing an allergic reaction, or increasing the risk of antimicrobial resistance. Therefore chitosan purity methods need to become more prominent.

As chitosan production moves to less chemically harsh and more sustainable approaches it remains increasingly important that contaminating substances which may cause harm to consumers is properly investigated and monitored with regular testing. Hazard analysis and critical control point principles (HACCP) should be followed preventing contaminating unsafe material from being in contact with processed material and any available confirmatory tests for purity of chitin and chitosan should be performed regularly.

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Chapter 5. General discussion, conclusions and future studies

5.1 Economic and Societal Aspects

There is an increasing demand to supply sufficient food and materials for a growing global population (UN, 2021a; UN, 2021b). Agriculture currently uses a significant proportion of land and water (UN, 2021b; UN, 2021c). Alternative methods for supplying foods and materials without the impact on land use and water usage have developed over recent years and are becoming more important. A further shift away from the harvesting of natural resources towards the use of cultivated resources is occurring which may help satisfy the reduction in land and water use and has the potential to provide enough food for the population.

As climate change continues and we see an increasing number of extreme weather events, our dependency on natural harvesting becomes more problematic, putting strain on already challenging conditions in low income countries with increasing proportions of the population experiencing hunger and malnutrition (WFP, 2021; UN, 2021d). Some cultivated methods do not have dependency on seasonality and can be geographically independent provided they have the appropriate infrastructure to provide the necessary resources. These methods could further help to provide resources when crop harvests fail due to climate change events.

Furthermore with the increasing need for protein for an increasing population we need to increase our efficiency, and ensure we are making full use of cultivated foods and materials that we already invest greatly in. In order to do this there has been increasing emphasis on circular approaches which recover value from waste streams.

Anaerobic digestion is being developed for supplying biomethane, as a sustainable replacement for natural gas derived energy, from waste streams such as sewage and agri-food residues (Innard and Chong, 2022). Insect bioconversion and more widely insect farming has been studied and implemented as a mechanism towards a circular economy. It provides value from waste streams to establish a protein source which can contribute towards food security. These methods can contribute towards several of the UNs Sustainable Development Goals including increased food security, improved waste management and direction towards a circular economy, positive effects on the environment, generating jobs, encouraging sustainable development, and improving human wellbeing.

In particular, insect bioconversion has received a great deal of attention in recent years with commercial developments in developing countries. Insects are a good source of revenue in developing countries where there is a reliable demand for them. When sold at markets they generally achieve good prices, better than crops, and sometimes meat and fish (van Huis *et al.*, 2013).

The growth of the alternative proteins sector is set to increase to up to 33% of the global protein market by 2054 (Chaalala, Leplat and Makkar, 2018). Insights (2020) predict that the edible insect market will be worth more than 710 million USD by 2026. It is an opportunity to diversify economies as it is reliable and not dependent on seasonality. On a small scale the farms can have a positive impact on the livelihoods of small livestock and fish farmers by diversifying their feed supply options and supplying an additional source of income.

In order to further expand the output of insect farms, one option is to increase the size of the farms by expanding vertically, this is beneficial as it uses the same amount of land as the smaller scale farm. Similarly, vertical farming is being developed for cultivating plants, where crops are grown indoors under artificial lighting conditions. Vertical farming has the potential to reduce water usage and can use hydroponic systems without soil. Vertical farming could also remove seasonality and external conditions which induce variability such as pests and disease and therefore reducing consumer risk and environmental pollution from pesticide and herbicide residues.

However, while vertical farming provides foods that are more acceptable to western society and plant based diets, there may be increased start-up costs associated with expensive technology and costs for increased energy usage. But in recent years, like with insect farming, there has been growing interest. This has been from higher income countries and has seen development in cities like Dubai (Emirates, 2023) and Copenhagen (Castillo, 2021).

Both vertical farming and insect farming can further contribute to the sustainable development of increasing urbanisation as they can be built anywhere, use minimal land and water, and contribute to food supply. In addition they could minimise the carbon footprint introduced through importation and transportation of resources through local production. Another sustainable mechanism for this is the use of aquaculture and fisheries, while this is more dependent on external weather and geography, it is useful for supplying resources in abundance. Seaweed aquaculture has the opportunity to sequester carbon from the

atmosphere and supply useful materials such as alginate and carrageenan which have wide spanning applications.

The ability to increase feed supply with minimal land and water usage, increases opportunities in other areas too. Insects are a natural food source for fish and some animals which could increase production of seafood and efficient proteins from animals like poultry. Furthermore the development of sustainable aquaculture and fisheries also increases supplies of protein and materials such as chitin. Mahtani chitosan is a company based in India which generates over 350 metric tonnes of chitin on an annual basis from wild caught shrimp waste (Chitosan, 2022). Primex is another chitin based company in Iceland which generates products from shellfish waste for use in different applications (Primex, 2022).

Insects are also a source of chitin. Insect farming could generate fast, reliable, sustainable chitin rich waste which, with development of processing, can be economically beneficial for a wealth of applications as previously described in Chapter 1. But the development of chitin waste into high value materials often requires technology which is not available in lower income countries where insect farming is currently more frequently employed.

One of the main barriers to the development of insect farming in higher income countries is the association of insects with dirt and disease and the consumers perception of this (Harvey, 2019). This negative association is not present in many low income countries as there is historical entomophagy within the population. Veolia have set up a farm in Malaysia (Veolia, 2023), Wilderspin and Halloran (2018) highlight the success of the establishment of insect farming in countries with historical entomophagy such as in Africa and South East Asia and indicate that the success may in part be due to the minimal use of regulation, legislation and policy (RLP) around the rearing of insects as they are already viewed as a food. This lack of framework can have positive and negative implications for sustainable insect farming (Wilderspin and Halloran, 2018). The lack of RLP can benefit farmers and collectors because they are not required to follow standards which can increase costs. However, there are negative implications also associated with limited RLP which means that the financial incentive rises and there are no standards to follow. This means shortcuts can be taken to increase profits that may not be sustainable such as increasing the harvest of insects in a way that may have negative environmental effects (Wilderspin and Halloran, 2018).

It has been reported that restrictive European Union regulations have limited investment in the insect farming industry (Wilderspin and Halloran, 2018). But growing demand for animal

source foods and declining availability of land means that there is a need for alternative proteins with minimal environmental and economic costs. More recently the European Union has permitted some insect species into the food market such as *Locusta migratoria* (EU, 2021). Therefore there are opportunities developing for insect farming within European countries. In the future this means that there will be more opportunity to further enhance the circularity of insect farming with valorisation of the waste generated. In addition other decomposing and saprophagous taxa could become involved in these mechanisms such as fungi.

Sustainable circular mechanisms which valorise waste streams are themselves going to produce waste materials. Being able to further utilise these waste streams will enhance the circularity of economies and could provide further jobs, income and increase the supply of biologically derived materials into biotechnological applications. Identifying points at which sustainable practices overlap and may complement one another will therefore be significant in developing further efficiencies in the future. Therefore multidisciplinary networking collaborations will be increasingly important.

The commercial developments of insect bioconversion could increase in value by taking advantage of their own waste streams and optimising their products into fractions of lipids, proteins and chitin (Caligiani *et al.*, 2018; Smets *et al.*, 2020; Wakefield, Mason and Dickinson, 2021). FERA science limited, a company which has invested greatly in insect bioconversion research and development (Limited, 2023), sought an interest in producing value from this chitin rich waste. Chitin rich materials require separation to extract the individual components. Biorefinery of chitin rich sources has recently become more appealing. Utilising chemical methods so far has been adequate but more sustainable approaches which minimise chemical waste such as fermentation with different enzyme cocktails could help enhance the sustainability of valorising a waste stream from a mechanism already utilising a waste stream and therefore enhance value and efficiency further.

This project was funded by FERA Science Limited to characterise chitosan from the black soldier fly for biobased food contact material applications. This is because chitosan could be profitable and has the potential to further satisfy Sustainable Development Goals. It can contribute to the development of materials which could be used as alternatives to plastics. It may be used as a flocculating agent in waste water treatment which could improve the

health and wellbeing of the human population as well as contributing to a reduction in pollution. In addition, it has antimicrobial properties which make it suitable for a range of applications which could contribute to minimising food waste and therefore improving food security, and improving the health and wellbeing of the human population.

5.2 Technical considerations

We planned to extract, characterise and apply chitosan derived from black soldier fly for food contact applications. In order to do this the following **aims** and objectives were previously outlined:

1 Extract chitin from the black soldier fly

1.1 Extract chitin comparing two chemical based methods on a small scale

1.2 Increase the scale of the extraction to provide chitosan on a gram scale.

2 Deacetylate chitin and characterise the chitosan

2.1 Degree of acetylation characterisation

2.2 Develop an affordable method for measuring molecular weight of chitosans in the lab

2.2.1 Calibration of the specific system developed

2.3 Characterise the molecular weight of chitosans

3 Assess the antimicrobial activity of chitosan

3.1 Develop antimicrobial susceptibility tests to *Escherichia coli* and S*taphylococcus aureus*

3.2 Measure the antimicrobial activity of chitosan to *Bacillus subtilis*

3.3 Measure the antimicrobial of chitosan against a selection of foodborne bacteria

3.4 Compare the responses of a panel of *Bacillus* reporter strains to different chitosans

4 Apply chitosan as a coating material to a food to reduce spoilage

4.1 Collect information regarding the perishable nature of foods using HorizonScan (2021)

4.2 Construct a narrative towards a specific food group utilising information gathered from HorizonScan (2021) and antimicrobial susceptibility tests

4.3 Measure the effects of different coatings on a food using total viable counts (TVC), thiobarbituric acid reactive substances (TBARS) and pH

4.4 Measure the effects of a chitosan compared to the control to determine if there are significant differences in TVC TBARS and pH values.

These were set to provide a focus and a structure to monitor and reflect on progress.

5.2.1 Extract chitin from the black soldier fly

5.2.1.1 Extract chitin comparing two chemical based methods on a small scale

Chitin was extracted from black soldier fly prepupae via a conventional chemical extraction method where whole powdered prepupae were defatted with hexane, demineralised with acid and deproteinated with alkali.

A method previously highlighted in the literature utilising deep eutectic solvents resulted in a poor outcome which did not function as an appropriate extraction method. On reflection this DES method may have more purpose in dissolution of recalcitrant material rather than as an extraction procedure.

Fermentation instead appears a more promising green prospect. Using novel green solvents for enzymes which require specific conditions that make their activity more difficult to achieve could also be developed.

The conventional method proved reliable in providing a yield of chitin which was comparable to previous reported values for black soldier fly in the literature. Similar conditions are used for different insects, so further optimisation towards specific substrates is needed. There is also a need for further development of methods to properly analyse the purity of chitin. Being able to measure the quality of chitin chains could be useful for comparing deacetylation reaction conditions.

5.2.1.2 Increase the scale of the extraction to provide chitosan on a gram scale

The stability of chitin in extraction conditions needs to be better understood. Using chemicals may have impacts on degrading the polymer and reduce the yield available. Our efforts to scale up chitosan production for use as a food coating, did not reach sufficient quantities to produce a food coating after use in previous experiments.

Purity assessment is essential for accurately determining a yield, if more time was available it would have been useful to further analyse the purity of the chitin extracted from BSF before deacetylation. Differences in the chitin available from different life stages would be useful once reliability is achieved with extraction procedure, quantification methods and purity assessment. Chitin quantification reliability would be greatly improved with extraction procedure optimisation for different substrates and accurate purity analysis methods.

5.2.2 Deacetylate chitin and characterise the chitosan

5.2.2.1 Degree of acetylation characterisation

Commercially available chitosans differed from certificate analysis. This brings into question the stability of DA over time and questions whether the deacetylation of a batch is not homogenous and more accounting for variability is needed. Further to this, understanding if or what are the causative factors that degrade chitosan could help understand its stability and variability. It would also help in understanding the biodegradability for its biotechnological applications such as food coatings. BSF was thoroughly deacetylated with the method we used and more deacetylated than any of the other commercially available samples.

5.2.2.2 Develop an affordable method for measuring molecular weight of chitosans in the lab

5.2.2.2.1 Calibration of the specific system developed

Temperature stability requires equipment which is expensive. Monitoring the temperature highlighted that the temperature remained relatively stable throughout the viscosity measurements of chitosans but was not perfect. This shows that even on a limited budget chitosan molecular weight characterisation by viscometry is feasible.

5.2.2.3 Characterise the molecular weight of chitosans

Chitosan from BSF MW distribution has not been previously evidenced and showed a broad range.

Aggregations make up a small component of the chitosan sample. At higher chitosan concentrations in solutions aggregation behaviour may influence application performance

and introduce further variability which may not be possible to fully explain without detailed method descriptions.

Developing the viscometry method was not time efficient but did prove budget effective. It was clear that even without perfectly stable temperature, the differences in the chitosans we used could be evidenced.

Acid hydrolysis and dialysis beforehand could separate differently sized fractions. Stability studies could also be useful to understand what causes molar mass to degrade, if it does. It is important that this includes the aggregation behaviour as this could falsely indicate differences in molecular weight if not cautious.

5.2.3 Assess the antimicrobial activity of chitosan

5.2.3.1 Develop antimicrobial susceptibility tests to Escherichia coli and Staphylococcus aureus

Adapting antimicrobial susceptibility tests to chitosan proved challenging, but it was successful after several optimisation experiments. Broth inoculation was not possible without chitosan causing precipitations. Identifying precipitants would be useful with more time. This would help in further method development for chitosan antimicrobial susceptibility.

Using enough material to provide a response was another challenge. Applying solvent cast films had limited effect therefore agar well diffusion was adopted as the main method which permitted loading more sample onto plates.

5.2.3.2 Measure the antimicrobial activity of chitosan to Bacillus subtilis

Bacillus subtilis screened through agar well diffusion showed good inhibition which enabled use of the *Bacillus* reporter strains effectively as all chitosans were inhibiting *Bacillus* reliably and the background control was not.

5.2.3.3 Measure the antimicrobial activity of chitosan against a selection of foodborne bacteria

In combination with HorizonScan, the antimicrobial susceptibility testing significantly facilitated a direction toward a food group for Chapter 4 after identifying *Pseudomonas* and *Salmonella* inhibition. Further work is needed to optimise antimicrobial susceptibility testing for chitosans against fastidious organisms as the blood aggregation made it challenging to read plates for zones of inhibition.

Enterococcus faecalis was resistant to inhibition and this could mean lactic acid bacteria as a whole may have resistance. The development of resistance of gram positive *Staphylococcus aureus* has already been highlighted in previous literature (Raafat *et al.*, 2017) and further work is needed to understand the risk of inducing the multi peptide resistance factor.

5.2.3.4 Compare the responses of a panel of Bacillus reporter strains to different chitosans

Comparing the responses of *Bacillus* reporter strains proved significant because not all chitosan samples provoked the same response. It evidences chitosan characteristics such as degree of acetylation, and/or molar mass have different effects and may affect the way bacteria respond. This further emphasises the importance of characterising chitosan. Carbohydrate microarrays may help in understanding if and how chitosan attaches to cell surfaces. In addition, chitin deacetylases can modulate the degree of acetylation which could have significance for the antimicrobial activity of the molecules.

5.2.4 Apply chitosan as a coating material to a food to reduce spoilage

5.2.4.1 and 5.2.4.2 Collect information regarding the perishable nature of foods using HorizonScan. Construct a narrative towards a specific food group utilising information gathered from HorizonScan and antimicrobial susceptibility tests.

HorizonScan made it easy to compare differences between a wide range commodity groups and understand the major hazards present for different groups. This could be more useful and further developed as a method for predicting future issues concerning food security.

5.2.4.3 Measure the effects of different coatings on a food using TVC, TBARS and pH

The different coatings used produced considerably different effects on the target food. Some showed a slowing effect on the increase of TVC over time. Layer-by-layer assembly was most effective at limiting the TVC. More replicates were necessary but lab throughput capacity was limited. Using more replicates in a high throughput environment could help in understanding the differences evidenced here. Understanding the microbial composition that makes up the TVC could explain the changes further too.

5.2.4.4 Measure the effects of a chitosan compared to the control to determine if there are significant differences in TVC TBARS and pH values

A significant impact on TVC was measured. But the TVC caught up with the control later into the experiment. Target food microbial composition variability, multiple sources and larger replicate scale need assessing. Challenge experiments where they test changes in temperature could also be interesting.

Modified atmosphere packaging has been studied in combination with chitosan and would be the main rival to utilising coating materials on the target food. Modified atmosphere packaging has previously been highlighted to be a risk at increasing the pathogenic bacteria. Chitosan antimicrobial performance against higher graded hazardous organisms could be interesting. In particular, those that would thrive more so under the conditions induced by the coating could be studied in greater detail such as *Clostridium* species and those that are commonly associated with the target food such as *Campylobacter* species. Characterising the mechanical and physical properties of the coatings would be useful to further understand the link with performance.

5.3 General concluding points

There is a need for a framework for the production and application of chitosan to ensure consumer safety and traceability of manufacturer. Guidelines for characterising chitosan's degree of acetylation and molecular weight distribution would be useful. In addition more attention is needed to assess the contaminant risks associated with using different chitosans. Chitosan's main biological origins are sources of common allergenic proteins. HACCP needs to be followed to ensure allergen safety and confirm chitosan is not being contaminated with unpurified biological materials. Safely applying chitosan would further benefit from more research to understand contaminant risks from non-intentionally added substances and to develop more methods for purity analysis. Biodegradability and environmental impacts of using more of these materials needs to be explored.

Multi peptide resistance factor has not been conclusively ruled out and there are still unknowns regarding the mode of action. *Enterococcus* resistance to chitosan in antimicrobial susceptibility testing needs further study. This needs exploring further prior to application to ensure it is not creating more risk than benefit. If the mode of action is properly understood, it would make application a lot safer and could tailor the applications more appropriately. Greater attention is needed in the direction of safely applying biopolymers in contact with foods. Challenge studies could also be trialled to test the performance of using these materials.

Composite and nanomaterials show promise for future applications. The layer-by-layer assembly we measured showed slower total viable counts. Combining materials for synergistic effects is an appealing area. Modern methods to make materials more active could include utilising bacteriophages to target specific pathogens that may be significant risks. Smart materials which respond to changes in conditions would be useful and could utilise different polyelectrolyte structures such as coacervates and other encapsulations like nanoparticles for delivery methods that can penetrate substrates. Adding nutritional value by incorporating vitamins is an added extra that could also add further purpose but it is important that a competitive performance to the fossil fuel derived plastics being replaced is achieved too.

Utilising recalcitrant biologically derived materials is becoming more important. Chitosan is a common product of arthropods which can come from sustainable fisheries and aquaculture practices, and insect bioconversion. Establishing greater quantities of biopolymers from

sustainable practices is likely to enhance development and performance of biopolymer based materials which will eventually outcompete fossil fuel derived plastics in the near future.

These processes on a global scale can help to reduce food waste through enhanced preservative effects and nutrient cycling to provide more protein, increasing food security. Reducing the use of fossil fuel derived plastics can help to minimise their pollution effects and thereby make a positive contribution to the UNs Sustainable Development Goals, lessening the impact of the three crises on Earth and encouraging a circular bioeconomy.

5.4 References

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Appendix A

6.1 HorizonScan

Table 23 – Key for the abbreviations of different commodity group hazard types as they appear in Table 24a, b, c.

Key	
A/S	Adulteration/substitution
AC	Aerobic colony counts
AO	Altered organolepsis
B	*Bacillus cereus*
C	*Campylobacter*
CO	Sudan dyes/colours
COL	Coliforms
CY	Cyanide
DI	Dioxins
E	*Escherichia coli*
ENT	Enterobacteriaceae
F	Fungal moulds and yeast
FB	Foreign bodies
FD	Fraudulent documentation
HEP	Hepatitis A
HIS	Histamine
HM	Heavy metals
IN	Infestation – insects etc
IR	Import refusal
IRR	Irradiation
L	*Listeria*
M	Morpholine
MO	Mineral oil
MY	Mycotoxin
ND	No data
NOR	Norovirus
NT	Other natural toxicants
OC	Other organic contaminants
OG	Other genetically modified organisms
OTH	All others
PAH	Polycyclic aromatic hydrocarbons
PE	Pesticides
PI	Processing issues
S	*Salmonella*
S.t/e	*Salmonella typhimurium/enterica*
SU	Sulphites
UA	Unauthorised/undeclared additives
VD	Veterinary drugs

Table 24a- Commodity groups compared against top five reported hazards within two to four weeks of 21st July 2021 according to HorizonScan data. Table 23 provides the definitions for the abbreviations of the different hazard types. Green background equalled a microbiological factor and orange background equalled a decomposition factor. The numbers in the columns are references to certain food safety issues.

Commodity group	Top five hazards					Risk	Emerging issues	Increasing issues	Fraud issues	Supplier check
Meat and meat products (excluding poultry)	S	L	E	FB	AO	1594	8	27	890	4528
Poultry meat and poultry products	S	S.t/e	VD	L	C	760	1	20	205	1327
Feedstuff	S	MY	A/S	ENT	IR	760	10	9	352	627
Fresh herbs	S	PE	IR	E	NT	1036	4	5	61	1154
Products of animal origin (dairy)	IR	L	E	FB	F	776	22	16	258	4723
Other seeds - sprouted	IR	S	PE	L	E	67	0	1	2	238
Pulse products	PE	F	FD	AC	DI	4	1	0	2	4
Oilseeds	MY	S	PE	IR	IN	414	15	7	184	2141
Spices - fruits and berries	IR	S	MY	CO	MY	238	0	2	83	1968
Herbal infusions - other	IR	S	NT	PE	UA	60	1	3	25	294
Sugar plants	IR	F	DI	PE	AO	2	0	0	0	5
Leaf vegetables	PE	IR	S	L	E	909	2	3	3	792
Brassica vegetables	PE	IR	E	L	FB	636	2	1	1	439
Spices - gums and resins	IR	PE	AC	S	PI	3	9	0	0	29
Stone fruit	IR	PE	L	SU	S	431	1	0	2	284
Oilseed products (other than oil)	MY	IR	B	FD	S	127	2	0	40	423
Spices - bark	IR	HM	F	FB	B	13	0	0	4	126
Fruiting vegetables	PE	IR	S	MY	AO	1965	5	5	39	4300
Tree nuts (shelled or unshelled)	MY	IR	S	CY	FD	534	7	1	214	1841
Spices - mixtures and blends	IR	CO	S	MY	CO	198	6	3	65	1572
Bulb vegetables	IR	PE	S	SU	SU	165	3	1	11	500
Spices - seeds	IR	MY	S	PE	NT	140	3	1	53	544
Spices - aril	IR	SU	F	A/S	MY	6	0	0	1	6
Seafood	IR	VD	HM	L	S	1513	16	20	645	11601
Legume vegetables	PE	IR	FB	L	E	629	0	0	5	1153
Fungi	IR	PE	IRR	S	L	164	0	2	20	1172
Citrus fruit	PE	IR	M	L	AO	580	1	1	3	385
Spices - roots and rhizome	IR	PE	MY	S	SU	123	1	1	8	386

Table 24b- Commodity groups compared against top five reported hazards within two to four weeks of 21st July 2021 according to HorizonScan data. Table 23 provides the definitions for the abbreviations of the different hazard types. Green background equalled a microbiological factor and orange background equalled a decomposition factor. The numbers in the columns are references to certain food safety issues.

Commodity group	Top five hazards					Risk	Emerging issues	Increasing issues	Fraud issues	Supplier check
Other processed starch foods	IR	MY	MY	S	PE	87	0	0	4	409
Other seeds - unprocessed (excluding pulses)	MY	IR	PE	S	FD	81	2	2	38	348
Herbal infusions (dried) - leaves	IR	FD	PE	S	NT	25	0	0	25	293
Miscellaneous fruit - inedible peel large	PE	IR	OG	UA	COL	465	4	2	5	982
Pome fruit	PE	IR	AO	SU	L	438	0	0	0	155
Herbal infusions (dried) - roots	IR	PE	IRR	UA	S	65	0	0	4	150
Herbal infusions (dried) - flowers	PE	IR	FB	IN	S	25	1	0	0	66
Processed foods	IR	FB	CO	UA	UA	2522	29	46	610	19509
Cereals - processed	IR	UA	FB	PE	UA	1219	14	18	251	8367
Chemicals used in foods	IR	CO	FB	VD	HM	48	4	0	26	551
Berries and small fruit	PE	IR	NOR	HEP	FB	1396	4	0	12	892
Dried fruits and vegetables	IR	MY	MY	SU	SU	742	7	5	66	2271
Beverages	IR	AO	A/S	CO	FB	679	8	6	626	5678
Cereals - unprocessed	IR	PE	MY	AO	OG	635	8	3	43	2665
Canned foods	PI	FB	HIS	FD	IR	331	10	1	142	786
Root and tuber vegetables - temperate	PE	IR	HM	SU	MY	308	0	2	5	559
Stem vegetables	IR	PE	FB	SU	HM	266	0	1	3	957
Products of animal origin (other)	VD	IR	A/S	FD	PI	255	5	3	221	806
Tea, coffee, cocoa, carobs	PE	IR	AO	MY	FD	242	2	0	47	1290
Pulses	PE	IR	CO	FD	FB	231	4	2	44	576
Food and Dietary supplements	IR	UA	UA	OC	PE	197	9	12	268	2407
Miscellaneous fruit - edible peel	IR	PE	FB	CO	SU	167	0	2	7	777
Foods for infants and young children	IR	FB	PI	UA	PE	142	2	0	30	628

Table 24c- Commodity groups compared against top five reported hazards within two to four weeks of 21st July 2021 according to HorizonScan data. Table 23 provides the definitions for the abbreviations of the different hazard types. Green background equalled a microbiological factor and orange background equalled a decomposition factor. The numbers in the columns are references to certain food safety issues.

Commodity group	**Top five hazards**					**Risk**	**Emerging issues**	**Increasing issues**	**Fraud issues**	**Supplier check**
Oils and fats	IR	CO	PAH	PE	PAH	134	0	4	158	800
Miscellaneous fruit - inedible peel small	PE	IR	SU	SU	CO	126	0	1	1	209
Root and tuber vegetables - potatoes	A/S	FD	PE	IR	FB	108	0	0	89	159
Root and tuber vegetables - tropical	PE	IR	CY	SU	UA	102	1	0	3	392
Sugars	IR	CO	UA	FB	UA	34	3	0	24	289
Oilfruits	IR	PE	MO	SU	FD	25	0	0	1	48
Novel foods	IR	UA	UA	FD	UA	6	0	0	5	1050
Spices - buds	IR	FB	MY	AO	PE	3	1	0	0	26
Spices - flower stigma	IR	CO	A/S	CO	OTH	2	0	0	1	14
Hops	ND	ND	ND	ND	ND	0	0	0	0	0