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College of Science, Engineering and Food Science



Assessment, Development and Optimisation of Packaging Systems for Cheese Products using Smart Packaging Technologies

A Thesis submitted in the Fulfilment of the Requirements for the Degree of Doctor of Philosophy

Presented by

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Under the supervision of,

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January 2016

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Declaration

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

Signature: _____

Karen A. M. O' Callaghan

Certified by: _____

Prof. Joseph P. Kerry

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This thesis is dedicated to my friend, Colm Johnson.

‘In the end; we only regret the chances we didn’t take, relationships we are afraid to have and the decisions we waited too long to make.’

List of Abbreviations

AA	Acetic acid
AF	Atomic force
AFM	Atomic force microscopy
ANOVA	Analysis of variance
As.A	Ascorbic acid
B. cereus	*Bacillus cereus*
BASB	Benzoic acid solubilisate
BASN	Benzoic acid solution
BHA	Butylated hydroxylanisol
BHT	Butylated hydroxyltoluene
B-LMWC	Bulk low molecular weight chitosan
CFU	Colony forming units
cm	Centimetre
cP	Centipoise
CUR	Curcumin
Da	Dalton
DNA	Deoxyribonucleic acid
EAA	Ethylene acrylic acid
E. coli	*Escherichia coli*
EVA	Ethylene vinyl acetate
EVOH	Ethylene vinyl alcohol
FFS	Film forming solution
FNA	Feruloylnoradrenaline
g	Gram
g	Gravity
GM	Genetic modification
GMO	Genetically modified organism
HALS	Polymeric hindered amines
HPMC	Hydroxypropylmethylcellulose
HDPE	High density polyethylene
hr	Hour
kDa	kiloDalton
LLDPE	Linear low density polyethylene
LDPE	Low density polyethylene
LMWC	Low molecular weight chitosan
log	Logarithm
MA	Modified Atomsphere
MAP	Modified Atomsphere Packaging
Met.	Metallised
MHB	Mueller hinton broth
MHz	Megahertz
MIC	Minimum inhibition concentration
min	Minute

ml	Millilitre
mm	Millimetre
MMWC	Medium molecular weight chitosan
MPCA	Milk plate count agar
MPDH	Masters degree, postgraduate diploma, doctoral degree or higher doctorate
mV	Millivolt
n	Number
N	Normal
NaOH	Sodium hydroxide
NCIMB	National collection of industrial and marine bacteria
NFC	Near field communication
nm	Nanometre
NP-MMWC	Nanoparticled medium molecular weight chitosan
NP-ROSE	Nanoparticled rosemary
O	Oriented
O_2	Oxygen
PA	Polyamide
PB	Paperboard
PCL	Polycaprolactone
PDI	Polydispersity index
PDVC	Polyvinylidene chloride
PE	Polyethylene
PET	Polyethylene terephthalate
PFA	Further education and training course or an apprenticeship
P. fluorescens	*Pseudomonas fluorescens*
PGA	Polyglycolic acid
PHA	Polyhydroxyalkanotes
PHB	Polyhyroxybutyrate
PLA	Poly-lactic acid
PP	Polypropylene
PS	Polystyrene
PS	Primary or secondary school
PVA	Polyvinyl alcohol
PVC	Polyvinyl chloride
Q	Question
QR	Quick response
R	Correlation coefficent
RFID	Radio frequency identification
RNA	Ribonucleic acid
ROSE	Rosemary
rpm	Revolutions per minute
SABASB	Sorbic acid and benzoic acid solubilisate
SASB	Sorbic acid solubilisate
SASN	Sorbic acid solution
S. aureus	*Staphylococcus aureus*
SC	Semi-chemically prepared board

T	Test liner
TBX	Tryptone bile x-gluc
TDU	Third level certificate, diploma or university degree
TPP	Sodium tripolyphosphate
TSA	Tryptone soya agar
UV	Ultraviolet
WTK	White topped Kraft liner
v/v	Volume per volume
w/v	Weight per volume
YM	Compact dry yeast and mould
°C	Celsius
°	Degree
µl	Microlitre
µm	Micrometre
%	Percentage

Abstract

The principle objective of this thesis was to develop a package to improve packaging function for end use with cheese products. This objective was undertaken by focussing on the use of smart technology, inclusive of the areas of active, intelligent and nanotechnology. Research commenced by conducting a survey to evaluate consumer attitudes towards smart technologies as it was deemed important to gauge acceptance prior to development. Overall, respondents were accepting of the application of smart packaging technologies to cheese products. Intelligent oxygen sensor technology was employed on an industrial scale to evaluate the technical performance of commercial cheese packaging. Sensors demonstrated a high level of package containment failure, particularly with packs subjected to distribution. Natural substances (nanoparticled and non-nanoparticled) were assessed against cheese-derived cultures to determine antimicrobial activity for potential use as active packaging agents. Subsequently, the best performing agents, which were those that exhibited antimicrobial activity or an increased solubility, were combined to determine if synergistic relationships could be achieved. From this work, it was apparent that nanoparticled rosemary extract and non-nanoparticled chitosan (both low- and medium-molecular weights) demonstrated the greatest microbial inhibition. The success of non-nanoparticled chitosan led to the laboratory synthesis of nanoparticled chitosan. Manufactured nano-chitosan displayed similar antimicrobial effects to non-nanoparticled chitosan. Finally, agents possessing the greatest antimicrobial activity (non-nanoparticled chitosan, nanoparticled chitosan and nanoparticled rosemary) were individually incorporated into HPMC-based films and the efficacy of these films determined via cheese application. An inhibitive microbial response was achieved, particularly when nanoparticled films were employed. This

thesis successfully demonstrated the acceptance, need, operation and viable application of smart technologies to cheese products.

Keywords: Cheese, Packaging, Smart, Active, Intelligent, Nanoparticles, Consumer Acceptance, Optical Oxygen Sensors, Chitosan, Rosemary.

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CHAPTER 1 - Literature Review

Cheese Packaging: Review of Traditional and Advanced Packaging Systems

1.1 INTRODUCTION

Cheese is an extensively popular food product due to its diverse taste and texture, its convenience as a ready-to-eat food and its versatility as an ingredient. Cheese is also a naturally nutrient-rich food product and is nutritionally acknowledged as being a good source of protein, calcium and many other essential vitamins and minerals. For many individuals, cheese is a staple product in the daily diet and to some extent, per capita cheese consumption is an important measure of dietary quality (Beijing Orient Agribusiness Consultant Ltd., 2011). In 2014, the top five countries in the world with the highest kg cheese consumption per capita were; France (26.7), Iceland (25.8), Finland (25.6), Germany (24.6), and Denmark (24.6) (CDIC, 2015). As can be seen from these figures, developed countries continue to have a higher level of per capita consumption, mainly because dairy products are considered to be premium and expensive forms of nutrition.

In 2013, 21.3 million tonnes of cheese and curd was produced, with 6.1 million tonnes exported globally (FAOSTAT, 2015). The biggest importers of cheese are; Germany, Italy, and the United Kingdom, respectively. Germany, Italy and the United Kingdom represent quite mature markets and consequently, population growth rates in these regions are generally low and per capita consumption is relatively static for most dairy products (Donnellan *et al*., 2011). However, the outlook is positive for the global cheese market as significant increases are projected in cheese consumption, with cheese consumption expected to rise to over 23 billion tonnes by 2019 (IDF, 2010). Dairy product consumption growth rates are highest in regions where consumption is growing from a low base such as non-traditional dairy consuming nations. Rapid economic growth, urbanisation, increased use of

refrigeration and the globalisation of the western diet are also contributing to this increase in dairy product consumption (Donnellan *et al*., 2011).

1.2 CHEESE

Cheese is believed to have originated as a product over 8000 years ago (Eugster *et al*., 2012), with the accidental discovery of curds and whey in ruminant stomach pouches used as containment bags for the storage and transportation of milk. The presence of rennet in the stomach-based containers caused the milk to separate into curds and whey, creating the first resemblance to cheese. Over the many centuries since its discovery, different eras and regions have aided in the development and improvement of its manufacture, which have helped evolve and shape what we know as cheese today. In particular, the invention of pasteurisation and processed cheese, mass yielding of rennet and the development of pure microbial starter cultures have led to large-scale production and export of cheese. Due to all of these advances, cheese popularity has soared, with industrial cheeses being the most widely available. Prior to this progress, cheese was considered a speciality food, produced on individual farms and was expensive to purchase.

Generally, cheese manufacture occurs in two stages; first, milk is transformed into curds and secondly, the curd is then ripened to a cheese. Milk is usually the most common raw material used in cheese manufacture, but cream and whey may also be used. The source of this milk can be derived from a variety of different species of animal (sheep, goat, buffalo and other animals), but cow's milk is customarily the most prevalent. Milk can be pasteurised to provide a safer cheese and prolong its shelf-life. However, pasteurisation can modify processes that occur during ripening, which can affect taste and texture. Raw milk cheese develops a stronger flavour sooner, though its profile can be inconsistent. Milk can be homogenised or standardised to provide cheese with varying fat contents or to alter cheese structure. Additives like annatto can also be added to the initial manufacturing step to pigment

the cheese. Milk is then acidified via the starter culture, indigenous microflora or by direct acidification and a coagulation method (acid, rennet, acid/heat or concentration/crystallisation) is applied. Once the coagulum is formed, a series of techniques are implemented to remove the whey, such as; cutting, cooking, agitation, cheddaring, pressing, and other syneresis-promoting processes. On reaching the desired composition and texture; moulding, salting and packaging are applied. Cheese is available in many shapes including; as a mass of moist granules, spreads, crumbed, cubed, shredded, sliced, balled, grated, or as a variety of sizes in whole forms (e.g. round, triangle, rectangle, square, log, loaf, oval crescent, cylindrical).

Fresh cheese may be consumed straight away. Fresh varieties or cheese with shortened ripening times are often considered to have a mild flavour and are regularly subjected to the application of additional flavourings. A variety of ingredients may be added (herbs, spices, condiments, fruits, vegetables or nuts) or a dressing applied. Smoking or aging in certain environments may also be used to impart flavour characteristics. Ripened cheeses are matured over a period of time which can be from weeks to years. Young cheese usually has a moist, softer texture with a milder flavour; a longer maturation period produces firmer, dry cheese with a stronger, more pronounced flavour. Ripening occurs in a number of ways - surface ripened, mould ripened (internally or externally), internally bacterially ripened, and is initiated by employment of; a starter culture, a non-starter microflora present in the starting materials and processing environment and rennet. During this time, constituent breakdown occurs, microbes grow, and the characteristic flavour, texture and aroma of the cheese develops. During ripening, certain varieties can grow mould as a chosen trait, which have complex and heterogeneous microfloral systems, such as surface- and blue-veined cheeses. This mould is supplemented into production,

either by adding to the initial ingredients or by being applied to the surface. Internal moulding is achieved by creating deliberate cracks within the cheese (lack of pressing of the cheese curd or via needling), which allows essential oxygen to enter to activate mould growth. Surface mould is developed by smearing the surface of the cheese regularly during maturation to encourage growth as a layer on the surface. Some varieties of cheese produce gas on ripening which contribute to a desired eye development and cheese flavour. The eyes begin to form when the cheese is about three weeks old; eye formation is controlled to some extent by regulating the temperature of the ageing room (USDA, 1972). The number of eyes formed depends on the rate of gas production by the bacteria and the ability of the cheese body to entrap the gas. Too much gas can cause the development of too many eyes or large cracks; too little gas production can cause a lack of eye formation. It is differences in the manufacturing and ripening process that create the colossal amount of diversity amongst cheeses.

There are hundreds of varieties of cheese, differing from one another with respect to their name, size, place of origin, or packaging; even though manufacture, flavour and texture may be quite similar. With such a vast number of cheeses available, many attempts of classification have been developed to characterise and group their traits (Smith and Nakai, 1990; Early, 1998; Fox *et al*., 2000; Croker *et al*., 2005 and others). The features used to help classify cheese, either singly or in combination, include; type of raw material used (milk, cream, whey), raw vs. pasteurised product, species of animal, country of origin, basis of manufacturing technique, type of starter culture used, method of coagulation employed, curd characteristics, precipitation of milk constituents, cooking temperature, pH, chemical composition of the cheese (fat, moisture, calcium content, etc.), texture, ripening characteristics or time, high

performance liquid chromatography profiles, proteolysis products produced, type of rind development, and, potential end use. For the purpose of this review, the traditional classification of cheese based on moisture will be used, as the moisture content of cheese often dictates the packaging format employed.

Fresh and soft cheeses are associated with high moisture contents as they are made to retain a high proportion of whey during manufacture and produce a curd that is soft, but holds shape. Some are eaten straight away and some are aged over a short ripening period of one to six weeks (Chapman and Sharpe, 1990). As a consequence of high moisture retention during manufacture, it is a highly perishable product with a short shelf-life. Additionally, softness and fragility of the product render it more susceptible to damage during distribution due to the stress of movement and handling. Therefore, these cheeses require more packaging to combat perishability and contend with environmental pressures. Examples of soft and fresh cheese include; Brie, Camembert, Feta, Ricotta, Quarg, Cottage cheese and many more.

Semi-soft and semi-hard cheeses vary in moisture content, with the curd being firmer than fresh or soft cheese. This category also includes; blue cheeses, smear-ripened cheeses and some gas-forming cheeses. The shelf-life of semi-soft and semi-hard cheeses is slightly more stable than soft cheeses, but spoilage occurs in much the same manner. Packaging requirements are less strenuous as the curd is semi-solid. Examples of semi-soft and semi-hard varieties are Mozzarella, Limburger, Gouda, Caerphilly, Roquefort, Haloumi, Munster, Edam, Provolone and others.

Hard cheeses usually have a moisture content which ranges from 30-45% and are subjected to high pressure during manufacture to give a hard, uniform, close texture (Fox *et al.*, 2000). They are usually ripened over a period of three months to over a

year, and as they age, they become firmer, crumbly and more pungent. Hard cheese has a longer shelf-life than soft cheese due to their reduced moisture content. Once opened, they can last just under a month, depending on the variety and storage conditions employed. Cheddar, Emmenthal, Gruyère, Gjetost, Jarlsberg, Colby, Leerdammer, Leicester, are some examples of hard cheese varieties.

Very hard cheeses are very dry, hard, grainy or crumbly in texture, with mature and more pronounced flavours. The moisture content of this category of cheese ranges from 26–34%, and is made from partly skimmed milk and starter cultures of thermophilic lactic acid bacteria, which they are ripened slowly, over a period of one to two years (Chapman and Sharpe, 1990). The low moisture and fat content, in addition to high-cook temperature and salting contribute to their longer keeping quality. Very hard cheeses do not require complex packaging as they have long shelf-lives. These cheeses are usually suitable for export, even to warmer climates. Examples include; Parmigiano-Reggiano, Asiago, Kefalotyri, Queso Anejo, Manchego, Pecorino Tuscano, Sbrinz and more, many of which are often retailed in grated forms due to their hardness.

Processed cheese is usually a smooth blend of one or more natural cheeses and other optional ingredients, which is usually inexpensive to produce. The manufacture of processed cheese is a good way to deal with poor quality cheese, cheese with minor defects and cheese trimmings from natural cheese production. The mixture is hot-filled into its packaging and cooled. Processed cheese is often presented in the form of; blocks, slices, triangles, logs, spreads, dips, pastes, sticks and numerous other forms, as it easily adapts to the shape of moulds and packages. Processed cheese is more convenient than natural cheese due to its high cooking temperature, low pH, addition of preservatives and packaging, which when combined contribute to a stable

product with a good keeping quality and a relatively long shelf-life. Cheese substitutes or imitation cheeses may be generally defined as products that are intended to partly or wholly substitute for or imitate cheese, and in which milk fat, milk protein, or both are partially or wholly replaced by non-milk-based alternatives, principally of vegetable origin (Fox *et al.*, 2000). These are generally used as ingredients, but when sold for consumption, they are packaged similarly to processed cheese products.

The processes applied to cheese during manufacture, such as; pasteurisation and other heat treatments, addition of acid or salting usually results in the decline of most microbials. Generally the more processes the cheese has undergone during manufacture, the more stable the product remains. Survival of microbials after these processes generally leads to deterioration in product quality and can, in cases, generate major safety concerns. Cheese can deteriorate via microbial growth (mould, yeast, bacteria and spore-forming entities), enzymatic reactions, oxidation, photo-oxidation, other chemical reactions which lead to the alteration of product taste/flavour, odour, appearance and texture, development of functional changes and a loss in nutritional value. This deterioration is caused by non-starter bacteria present initially in the milk or via contamination during production. The most common pathogenic and spoilage microbes associated with cheese include; *Bacillus, Clostridium, Escherichia, Lactobacilli, Leuconstoc, Listeria, Micrococcus, Pseudomonas, Salmonella, Staphylococcus, Streptococcus*, moulds, yeasts (Lucas, 2003; Ledenback and Marshall, 2009). Therefore, it is important to make the cheese as unfavourable to spoilage as possible. This can usually be performed by controlling the ripening process, subjection to constant refrigeration temperatures and through

the application of the most appropriate packaging system, which should ensure that the cheese product achieves its full shelf-life potential.

1.3 PACKAGING

The packaging industry is the world's third largest industry sector, next only to food and petrochemical industries (Manalili *et al.*, 2011). By the end of 2012, the global packaging market reached over €640 billion, with a projected annual growth of 4% per year, up to 2018 (Smithers Pira, 2013). Paper and plastic hold the majority share in the packaging market, with metal, glass and other materials making up the remaining proportion. More than half of all packaging applications are dedicated to food (51%), with the rest of packaging applications purposed towards beverages, pharmaceuticals, cosmetics and other sectors (Neil-Boss and Brooks, 2013).

Packaging is an economic process which must technically provide a product like cheese with containment, protection and preservation, thereby ensuring that the correct packaging materials and systems match and counteract the properties and challenges presented by the cheese, protecting it against physical and environmental damage, while maintaining quality well beyond the natural life of the product. Additionally, packaging must inform all who come into contact with the product, must be capable of promoting the product adequately and must provide convenience, wherever possible, in a manner that is legally and environmentally-acceptable. These packaging functions must be implemented to all packaging levels – primary, secondary and tertiary. Primary packaging includes all of the packaging which surrounds the retailed product, secondary packaging is employed to group primary packs together for ease of handling (e.g. corrugated boxes), and, tertiary packaging is used to collate secondary packs to facilitate transport and usually involves palletising (Emblem, 2012).

The shelf-lives of products, which is associated with the preservation function, can be severely limited by primitive or inadequate packaging and distribution systems. This is especially true for products being transported over long distances and through fluctuating climates. Therefore, it is important when choosing a packaging design for cheese to find a system that is fit-for-purpose, thereby maximising its function. The classification of cheese (fresh, soft, semi-soft, semi-hard, hard, very hard and processed) must be taken into consideration when choosing a package type, as ultimately, the level of moisture is a sound indication of what type of packaging should be used. Higher moisture cheeses are the least stable and therefore, require more complex forms of packaging. Prior to being packaged, most cheese products are subjected to a process, whether it be refrigeration, thermisation, pasteurisation, salting, wax application or other treatments, to extend shelf-life, however, these alone are not enough to maintain a safe and high quality product. The parameters which contribute to the stability of cheese are water activity, light, oxygen and temperature, and most of these factors may be controlled or prevented from affecting the cheese through the correct application of fundamental, and some advanced packaging methods. Good cheese packaging is required to prevent the product from drying out, but also needs to prevent moisture gaining entry from the external environment to the product, which would increase water activity and potentially accelerate microbial growth. Depending on the cheese variety, either an excellent gas barrier or a selective gas barrier is required. A suitable barrier is necessary in circumstances where a modified atmosphere is employed, thereby preventing the entry of oxygen and minimising microbial growth and oxidation-driven reactions. Light can also initiate the oxidation of fat, even at refrigeration temperatures. Photo-oxidation of cheeses may be reduced by (i) minimising light exposure, (ii)

optimizing the packaging barrier, and (iii) improving headspace conditions (Mortensen *et al*., 2004). Achieving opacity via plastic-orientation, clever use of labelling systems or utilising certain smart packaging systems can prevent UV light from penetrating the package. Increased temperatures during distribution or storage can; accelerate microbial growth, cause texture deterioration and adversely affect the permeability characteristics of the packaging. Temperature is usually regulated by maintaining refrigeration conditions during storage, distribution and retail, although it can now be controlled by implementing smart packaging techniques. Both natural and processed cheeses are packaged in a variety of different formats, as demonstrated in Table 1, with a wide range of materials used.

Table 1
Packaging formats available for cheese products

Packaging Format	**Cheese Types**				
	Fresh/Soft	Semi-Soft/Hard	Hard	Very Hard	Processed
Aerosol Can	✓				✓
Bag (+ Brine or Whey or Water)	✓	✓			
Collapsible Tube	✓				✓
Cylinder Tube	✓	✓	✓	✓	✓
Foil Brick (+ Lamination) (+ Paperboard box)	✓	✓	✓	✓	✓
Glass Jar	✓	✓	✓	✓	✓
Grip and tear pack - Casing	✓	✓			✓
Individual Wraps (e.g. Cheese slices)					✓
Lamination	✓	✓	✓	✓	✓
Loose Plastic Wrap - one layer or laminate (+ Paperboard sleeve)	✓	✓	✓	✓	
Parchment (+ Paper sleeve or Wooden box surround)	✓	✓			
Pouch Bag	✓	✓	✓	✓	✓
Squeezy Bottle	✓				✓
Surface Wax (+ Net or Rope)		✓	✓	✓	✓
Metal Box				✓	
Metal Can	✓				✓
Tray	✓	✓	✓	✓	✓
Tub + Lid (+ Foil) (+ Brine or Whey or Water)	✓	✓	✓	✓	✓
Waxed or Laminated Paperboard (+ Wooden box surround)	✓	✓	✓	✓	✓
Cheese Pencil				✓	
Wooden Box	✓	✓	✓	✓	

(+) indicates additional treatment to the format such as medium or extra layer of packaging.

1.3.1 Traditional Packaging

1.3.1.1 Wax

Wax is one of the oldest forms of packaging employed for cheese, with different waxes being applied over the years, including; animal, vegetable, mineral and synthetic waxes. Originally, the cheeses would have been dipped manually into the wax or the wax brushed onto the cheese surface. However, to provide an even coating without over-applying, a spraying process is more efficient and most commonly employed, although other modern methods include casting, dripping or foaming. In general, waxes need to be food grade, have adequate melt and flow properties, have a good adhesion and short set-time to the cheese; accompanied by possessing high cracking- and scuff-resistance. In addition to protection and preservation, wax can offer a unique aesthetic and traditional appeal (useful for marketing purposes) and can help to develop taste, texture and rind characteristics. The performance requirements for cheese waxes differ considerably depending on the cheese variety being considered. Therefore, waxes can be specially formulated for certain applications. Specialised waxes like Paradip Nowax® (Invarson Inc.), are used instead of conventional waxes to allow for permeability of gases, as this enables the product to be used on cheeses which are ripening and producing gases and thus would normally have to be recoated several times (www.ivarsoninc.com). However, there are a number of disadvantages associated with waxing; duration of waxing increases production time; too high a temperature can cause cheese to disfigure; waxing alone is usually not sufficient and requires another form of packaging, often a residue remains on the cheese post-peeling and waxing is an energy-expensive process owing to keeping wax hot for successful application. Prospectively, it is edible and active waxes which are likely to dominate this area in the future. Brody *et*

al. (2001) stated that active substances have previously been incorporated into wax layers and packaged around cheese. However, for wax to be used as a packaging format on its own, then performance characteristics need to be improved.

1.3.1.2 Paper, Paperboard and Corrugated Paperboard

Paper is commonly used in cheese packaging, although it is not usually employed on its own. Paper, in some form, can be found at all three packaging levels; primary, secondary and tertiary, with the most common forms employed including; paper, parchment, paperboard (commonly referred to as cardboard) and corrugated paperboard (commonly referred to as fibreboard or boxboard). Paper and parchment are often used in laminates, in metallised applications, as overwrapping or as inserts between slices of cheese. Other paper formats employed in cheese packaging are paperboard sleeves or paper labels around the packaging to communicate product information. Paperboard/cardboard or corrugated paperboard/fibreboard are very common distribution container materials that are used in primary, but mostly in secondary and tertiary packaging, primarily as rigid boxes or cartons. The main advantages of paper products are; it is a sustainable material, biodegradable and compostable, low-cost and superior printability. Paper also possesses good structural properties and imparts tearability, but usually some form of coating or lamination must be applied to counteract its poor gas and moisture barrier properties and to overcome its lack of sealability. The future of paper use in cheese packaging is likely to remain stable, with the majority of its applications focussed in laminate construction and as protective packaging materials in secondary (handling and collation) and tertiary (transport) packaging. However, ongoing research is currently

investigating the chemical and physical manipulation of wood-derived nanocellulose fibres and some interesting and novel plastic-like materials have been produced that may have unique food and cheese packaging applications into the future (Future Market Inc., 2015). The short-term target for paper manufacturers should be to encourage light-weighting with paperboards, whilst maintaining or enhancing its properties. The functionality of paper should be expanded, with efforts focussed on transforming paper packaging into smart packaging forms. A German company, Keinenburg GmbH, have developed a cheap, environmentally-friendly beverage can made from cardboard (Astley, 2011). The can has the same technical properties of a metal can, but with the enhanced feature of keeping the contents cooler for longer. Whilst a can may not be the optimal shape of packaging for cheese, changing the form to a more suitable container may make it more relevant and useful in cheese packaging applications in the future.

1.3.1.3 Metal

Metal is not traditionally thought of being prolific in cheese packaging, although it is abundant in many forms. The formats in which cheese is packaged in metal include rigid and flexible forms; cans, boxes, aerosol cans, collapsible tubes, closures, laminates, coatings (vacuum metallising), or on its own as foil. Aluminium foils and vacuum metallised substrates are the chief use of metal in cheese packaging. Cans, aerosols, and collapsible tubes are most commonly employed in processed cheese packaging for foam and paste-like products. Metal boxes (most commonly aluminium, steel or tin) are usually used specifically for artisan cheese products or for custom applications. Metal packaging, depending on whether it is flexible or

rigid, can offer different advantages, but with both having an attractive glossy appearance and pure forms being recyclable. Both rigid and flexible forms can provide complete barrier properties and therefore can be described as hermetic. Flexible metal-based packaging can achieve this at very low thicknesses, but additionally have great dead-fold characteristics and are also very light in weight. However when compared to other forms of packaging, metal-based packaging is generally expensive, especially rigid forms owing to its weight. Thinner forms of light-weighted rigid and flexible metals can be easily damaged, owing to pin-holing, flex-cracking or tearing. Additionally, whilst pure metals are easily recycled, the recycling of metal-based laminates or vacuum metallised materials is more challenging, as the separation of metal from plastic or paper is very difficult. The future of metal use in cheese packaging is likely to reside with metallising and foil applications, with rigid metal packaging declining in use. Metal formats must become more versatile and increasingly cost-competitive. The advent of technologies that allow for the recovery of aluminium foil from flexible laminates, pouches and cartons is highly propitious. Enval have developed a continuous process for the complete recycling of laminate waste, recovering 100% of the aluminium present in the laminate (www.enval.com).

1.3.1.4 Wood

Wood is a rigid form of food packaging which has existed for many years, but its usage with cheese has been in decline for some time. Many different varieties of wood (pine, spruce, poplar, beech, ash, oak, and more) have been used in food packaging, usually as rigid boxes, as wood offers good physical protection, can be

easily stacked, is environmentally-friendly, can diffuse distinct flavours to food and is aesthetically attractive (often used to convey the element of history and tradition in the clever marketing of food and beverage products, like cheese). Foods packaged in wood are usually artisan or custom made, which exude a premium, fine food quality and uniqueness about them. However, the weight of the packaging may vary depending on whether solid wood or flexible veneer is used. Food-grade wood must be used in all food packaging applications. Plastic containers have largely replaced wood in most applications and will likely continue to do so in the future. Wood as a packaging material has a higher cost, and usually must be combined with another form of packaging to make its application economical. Additionally, it is often considered less suitable for packaging applications due to its absorbent nature and risk of splintering. However, some varieties of wood have been shown to possess antimicrobial characteristics such as pine, with wood extractives such as tannins and polyphenols being responsible for microbial reduction (Schönwälder *et al*., 2002). More research into wood as a naturally active packaging material is likely to increase its usage, but not at a mass level, and its long-term usage is most likely to be as presentation and storage boxes for primary and secondary packaging and for pallet construction for tertiary packaging.

1.3.1.5 Glass

The use of glass in the packaging of cheese is somewhat limited. It is used occasionally to contain processed cheese and some varieties of fresh and soft cheeses, and usually amongst artisan and small-scale producers of cheese. The benefits of packaging cheese in glass containers include; hermetic properties, inert

nature, strength, recyclability, good optical clarity, stability at high temperatures for hot-filling applications, and its premium retail value (as it epitomises quality, cleanliness, purity, history, and tradition). Glass containers provide convenience as they are typically resealable, often using a metal or plastic closure. Conversely, its disadvantages are; fragility, weight, noise generation and relatively high cost. However, if glass can be light-weighted while still performing adequately and at a reasonable cost, its use could be further reinforced in the future. Light-weighting reduces transportation costs and is more environmentally-friendly, but attaining even glass distribution while continuing to be safe and to support features like embossing and engraving. This kind of innovation could be applied to cheese packaging in the future, making glass packaging a more desirable form of packaging for cheese producers.

1.3.1.6 Plastics

By 2013, the worldwide annual production of plastics was approximately 299 million metric tonnes (Statista, 2015). They are the most frequently used packaging materials of all, whether employed solely or as a laminate or packaging component. Cheese packaging plastics can be found in both rigid and flexible formats and most commercially-employed food plastics can be found in nearly all mass-produced cheese packaging applications. The plastic formats used for cheese include; tubs, collapsible and cylindrical tubes, pouches, bags or sacks, laminates, films, sheets or wraps, closures, bottles, trays, cups and other containers. Plastics are used for cheese packaging applications because of their extensive list of advantages. They are not generally subject to corrosion, they are light-weight, possess a good strength to

weight ratio, cost-effective because of the ease and speed of manufacture, significant design freedom, rapid assembly time; generally good electrical insulators and multi-coloured (Alauddin, 1995). The properties of plastics are determined by the chemical and physical nature of the polymers used in their manufacture. The properties of polymers are determined by their; molecular structure, molecular weight, degree of crystallinity and chemical composition (Robertson, 2013). Plastic properties such as strength and barrier functions may be improved by physically orientating the polymer molecules, thereby allowing thinner sections of material to be used to achieve the same property characteristic as the thicker material. Each individual plastic material offers different properties and Table 2 lists the most commonly used plastic polymers employed for cheese packaging applications. However, the plastics industry is heavily integrated with the oil industry (Brydson, 1999). The production of plastic is highly dependent on petroleum as a source of raw material and energy. Plastics made from this source are not biodegradable, thereby presenting environmental problems.

Table 2
Most commonly used plastics in cheese packaging

Plastic	Abbreviation
Acrylic	
Ethylene acrylic acid	EAA
Ethylene vinyl acetate	EVA
Ethylene vinyl alcohol	EVOH
High Density Polyethylene	HDPE
Ionomer	
Linear Low Density Polyethylene	LLDPE
Low Density Polyethylene	LDPE
Polyamide	PA
Polyethylene	PE
Polyethylene terephthalate	PET
Polypropylene	PP
Polystyrene	PS
Polyvinylidene chloride	PDVC
Polyvinyl Chloride	PVC

1.3.1.7 Laminates

The purpose of a laminate is to bind two or more layers of materials together in order to combine the best of all the properties of the materials utilised at a reasonable cost in a single packaging structure. Laminates based on pure plastic constructions can be manufactured through co-extrusion, extrusion coating or extrusion laminating. Laminate materials can be tailored to product requirements and must be selected based on their compatibility with the product and the conditions the packaged product will experience throughout its lifetime. Structure, performance, barrier and appearance can all be enhanced using a laminate construction, as no one packaging material typically possesses all of the properties necessary for food packaging applications. Generally for cheese, a combination of plastic, paper, wax and metal is employed within a laminate. Additional tie layers (adhesives) may be necessary to yield adhesion between different material layers in the laminate. Table 3 presents examples of laminates which have been used to package cheese products.

Table 3
Examples of laminates that have been or are currently employed in cheese packaging applications

Laminates	Notes
Cellulose/OPP/PVDC	O denotes orientation.
Cellulose/PE/EVA	Cellulose is a biodegradable material.
Cellulose/Waxed PB	
EVA/PVDC/EVA	
Foil/tie/Glassine Paper	A tie is an adhesive layer.
Foil (coated or uncoated)/PB	PB is paperboard.
LDPE/PB/LDPE	
LLDPE/EVOH/LLDPE coated with EVA	
Met.OPP	Met. denotes metallisation.
Nitrocellulose/OPP/PVDC	Nitrocellulose - cellulose coated with nitric acid
Nitrocellulose/Waxed PB	
OPP/PVDC coated cellulose/EVA	
OPP/PVDC coated cellulose/Ionomer	
PA/EVA	
PA/EVA/PVDC + PVDC coated PET coated EVA (lid)	
PA/EVA/EVOH + PVDC coated PET coated EVA (lid)	
PA/EVOH/EVA	
PA/EVOH/LLDPE	
PA/LDPE	
PA/LLDPE	
OPA/LDPE	
OPA/LLDPE	
PA/PVDC/Ionomer	
PB/PVDC coated with cellulose coated with rubber modified wax	
PET coated PVDC/wax	
PP/PE	
PP/PE/PP/PVDC/EVA	
PP/PVDC/Regrind/PP	Regrind is recycled plastic.
PP/Regrind/tie/PP + PET/foil/PP (lid)	
PP/Regrind/tie/PP + PET/foil/PE/Sealant (lid)	Sealant is used as a barrier layer.
PP/tie/EVOH/tie/PP + Foil/Sealant/Closure	
PVDC coated cellulose/PVDC coated cellulose	
PVDC coated cellulose/PVDC coated OPP	
PVDC coated cellulose/PVDC/PA	
PVDC coated cellulose/Wax/Foil/Sealant/Starch polymer	Starch is a biobased polymer.
PVDC coated PET/EVA	
PVDC coated PET/Ionomer	
PVDC/EVA coated PA	
PVDC/EVA coated OPP	
PVDC/OPP/Acrylic	
PVDC/OPP/PVDC/EAA/met.PET/EVA	
Starch coated Nitrocellulose/Waxed PB	
Waxed PB	

Plastic abbreviations are defined on Table 2. Coating and laminating are differents methods of affixation.

A coating is denoted by statement and a lamination is indicated by '/'.

1.3.2 Advanced Packaging

1.3.2.1 Modified Atmosphere Packaging

Modified atmosphere packaging (MAP) can be defined as the enclosure of a food package in which the atmosphere inside the package is modified or altered to provide an optimum atmosphere for increasing shelf-life and maintaining the quality of the food (Robertson, 2013). MAP relies on using a mixture of gases, at concentrations different to those present in air, so as to retard deterioration processes and to assist in creating and maintaining pack shape. The types, combinations and concentrations of gases required in creating modified atmospheres depend on the foodstuff being packaged. Typically, oxygen, carbon dioxide and nitrogen may be used singly or in various combinations; all of which are colourless and odourless gases. Many studies have been undertaken to examine cheese held under MAP conditions, including; fresh and soft (Fedio *et al*., 1994; Westall and Filtenborg, 1998), semi-soft and semi-hard (Alves *et al*., 1996; Juric *et al*., 2003), hard and very hard (Taniwaki *et al*., 2001; Romani *et al*., 2002). Carbon dioxide, on its own or a mixture of carbon dioxide and nitrogen, are usually used for dairy products, including cheese in MAP applications. As oxidation and microbial growth can be an issue for cheese, then extremely low levels of oxygen are targeted within commercial packs. Carbon dioxide is a very expedient gas due to its bacteriostatic and fungistatic properties. It inhibits the growth of many spoilage bacteria, the degree of inhibition increasing with increasing concentration. The solubility of the carbon dioxide increases with decreasing temperature, therefore at lower temperatures, carbon dioxide antimicrobial activity is markedly greater (Robertson, 2013). This high solubility can also lead to package collapse if large amounts of carbon dioxide is applied, which can be desirable for hard cheeses. However, this can be unsuitable for soft cheese,

therefore, nitrogen is often used as a filler gas to counteract the package collapse caused by carbon dioxide dissolving into the water and fat components within the food. The selection of package, operation of the process and the function of storage conditions are all important for this application, otherwise all benefits associated with MAP are lost. In particular, the properties of packaging materials used in these MAP applications need to be carefully considered (permeability, structure, processability, sealability). Applying additional hurdles, such as refrigeration, use of active packaging systems etc. can help improve MAP performance, and ultimately extend shelf-life.

Another form of MAP is vacuum packaging, with nearly all ripened cheese products in retail packaged under either MAP or vacuum packaging (Brody, 1993). The modified atmosphere is achieved by applying a vacuum as opposed to a gaseous alteration. This packaging method deliberately utilises a vacuum to effectively evacuate the air surrounding the product. This removal of atmospheric oxygen prior to sealing, impedes the oxygen induced deteriorative processes described previously from occurring, leading to an extension of shelf-life. If a reasonable vacuum is obtained, the oxygen level can be reduced to less than 1% (Parry, 1993). Concurrently, as this low oxygen atmosphere is achieved, package collapse around the product occurs. Like MAP, the package material used for this application is critical as any function imposed is dependent on proper containment. Therefore, films or usually laminates with heat sealability and a low gas permeability, specifically against oxygen, are utilised. Vacuum skin packaging or shrink wrapping are similar methods to vacuum packaging, however the film is heated prior to application which ensures a tighter fit to the contours of the product contained. Again a vacuum may be employed in order to modify the environment surrounding

the cheese product. Often the aid of a support, like paperboard, is placed underneath the product. The process is more appropriate for harder cheese, as cheese with a high moisture content may deform with the force of package contraction achieved.

1.3.2.2 Biodegradable Materials

Food packaging waste has become one of the most pressing environmental issues facing human-kind globally in the 21st century, along with food sustainability and global climate change (Visiongain, 2014). Attempts to reduce, reuse and recycle food- and beverage-derived packaging has not had the desired effect of controlling packaging waste and the lack of debate on recovery *versus* landfill has only exacerbated the negative environmental impact of packaging waste. Food and beverage packaging materials are primarily offered via oil-derived plastics in single format or in laminate constructions. Consequently, these packaging materials are not biodegradable or compostable. Neither are they user-friendly in terms of reprocessing for new product applications, owing to the presence of possible residues, toxicants etc. Therefore, as the food industry (inclusive of the cheese packaging sector) is challenged to become more sustainable in nature and become less negatively impactful on the environment, there will be a need to examine packaging that has a greener image and which adds value to the waste stream generated from its use, for example, be biodegradable, compostable etc. It is evident that the potential market for bio-based packaging materials is enormous, although as it stands, bio-based renewable materials (excluding paper packaging) currently represent only ~2% of the packaging market (Johansson *et al.*, 2012). Therefore,

there is a requirement to investigate the development of new and novel ingredients for use as biodegradable packaging.

Ideally, when sourcing an alternative raw material, it should be readily available, food-contact-friendly and not cause environmental concerns. Natural polymers or polymers derived from natural monomers offer the greatest opportunities, since biodegradability and environmental compatibility are assured (Krochta and De Mulder-Johnston, 1997). Bio-plastics can be manufactured by various different techniques; directly extracted from natural materials (like polysaccharides, lipids and proteins), produced by 'classical' chemical synthesis from renewable bio-derived monomers, produced by microorganisms or genetically transformed bacteria, or modification using a disrupting agent (Petersen *et al*., 1999). Examples of these polymers, include; polyhydroxyalkanoates (PHA), bacterial cellulose, polyhydroxybutyrate (PHB), polycaprolactone (PCL), natural rubber, polyglycolic acid (PGA), polyvinyl alcohol (PVA), modified polyolefins, and more. Inedible waxes and coated cellophane are examples of biodegradable packaging that has been applied to cheese. Holm *et al.* (2006) assessed poly-lactic acid (PLA) packaging of semi-hard cheeses but found PLA had a high rate of oxygen transmission which caused lipid oxidation, resulting in the application of oxygen scavengers to reduce oxidation. Mainly owing to issues regarding processing, performance and cost, traditional plastics still dominate the marketplace. Also degradation of a bio-based polymer may result from the action of microbes, macro-organisms, photo-degradation or chemical degradation (Petersen *et al*., 1999). Therefore this means avoiding conditions, which are conducive to biodegradation, like light and moisture, in order to achieve a controlled lifetime and then biodegrade efficiently post use.

The future is likely to continue to focus on: exploring new and novel bio-polymer sources (which do not utilise ingredients which could be used as food sources) to produce bioplastics; reducing costs whilst scaling-up production; increasing communication with food producers, retailers, governments, and consumers regarding biodegradation; and improving processing and enhancing performance properties so as their functionality is equal to or greater than traditional petroleum-based plastics. These approaches will all help to increase commercial adoption and expand market access to such materials. Braskem, have developed 'green polyethylene' using ethanol, derived from sugarcane, which is recyclable and has the same properties and process abilities as fossil-based polyethylene (www.fkur.com). Also combining biodegradability with natural antimicrobials is increasingly becoming an area of interest. Specifically for cheese, research by the 'Biopack' project, investigated the development of a biopolymer-based packaging system that would provide an extended shelf-life via the incorporation of a natural biocide, for European cheeses (CORDIS, 2005). Plackett *et al*. (2006) evaluated modified and unmodified PLA and PLA-PCL, with incorporated nanoclays and/or cyclodextrins against cheese and demonstrated transparency, no risk of migration, mould inhibition and biodegradation; however, package permeability was an issue.

1.3.2.3 Edible Materials

Edible films and coatings are chiefly made up of polysaccharides, proteins, hydrocolloids, lipids and resins, which are usually derived from natural sources like plants, animals, and marine organisms. Edible films can be used singularly or together as bi-layers or as composites. These coatings can be clear, coloured or

opaque, flavoured or tasteless, but they must be food grade. Edible materials are applied in the same manner as wax applications, and like wax, are usually unsuitable for application to soft cheese as a continuous barrier on the surface cannot be achieved. Edible films and coatings can execute a range of functions; containment, barrier protection and preservation, improve appearance, anti-sticking properties and act as a carrier for additives, whilst being biodegradable. Additives like emulsifiers, plasticisers, surfactants, antimicrobials, antioxidants, flavour and aroma compounds, pigments, nutrients and other ingredients may be included to provide uniform coverage, improve strength or flexibility, keep components in solution, and enhance barrier or appearance, although addition of some of these ingredients may alter other film properties. Films also help establish a modified atmosphere or can be selectively permeable, which is useful for certain cheese-types. However, if the film or coating tears or erodes, all of the beneficial effect is lost, therefore, edible coatings usually require another layer of inedible packaging for sufficient protection.

Research has expanded into areas which may allow soft cheese to be packaged using edible packaging in the future. A patented edible film has been developed that can be applied to sticky or moist food products, like softer cheeses, to retain product shape, prevent moisture migration and allows for the single-serving of food components like cheese slices within a sandwich to be coated (Mayfield, 2000). The coating also allows for easy handling and uniquely possesses the tacky nature of cheese slices. Interest into the use of casein as an edible film would prove beneficial in cheese packaging as it may be extracted during manufacture. Researchers at the Agricultural Research Service found that combining casein with water and glycerol produces a water-resistant film that can be used as an edible coating for food products (Core, 2005). Harvard University have developed WikiCells which consists of a natural

edible membrane held together by electrostatic forces that can contain a liquid, emulsion, foam, or solid food substance such as cheese at any moisture level due to its significant water resistance (Edwards, 2012). The future of edible coatings is that they may be employed without the requirement of conventional plastics or with reduced levels of plastics required. This could be achieved through the use of multi-component composites and the integration of smart or active materials into edible packaging systems.

1.3.2.4 Nanotechnology

Nanotechnology deals with processing, generation and use of material particles which are nanoscale (1 nanometre is equal to 1 millionth of a millimetre). Particles at this size exhibit characteristics different to their bulk equivalents, mainly due to the increased surface area of materials obtained when reduced to a nano-structure. The technology improves mechanical, structural, performance, reactivity and barrier properties, and can impart a biocidal surface characteristic (Smolander and Chaudhry, 2010). These technologies can also offer transparency within the package and are excellent carrying systems, where the active component can be better dispersed and be more effective (López-Rubio *et al*., 2008). Therefore, due to this increased reactivity and dispersity, lower levels of the nanomaterial can be used with the same effect as the higher level of correspondent bulk material. Azlin-Hasim *et al.* (2015) demonstrated that silver nanoparticles within LDPE film reduced microbial growth and significantly extended the shelf-life of chicken, regardless of the concentration. Nanotechnology can be incorporated into traditional packaging or into advanced packaging systems (biodegradable, edible, active or intelligent) for cheese

packaging applications. Nanomaterials, including the nanocomposite Imperm®, have been incorporated into packaging to extend the shelf-life of cheese (De Azeredo, 2009; Silvestre *et al*. 2011). Nanoscale edible coatings can provide a barrier to gas and moisture and act as a carrier vehicle to deliver active agents, whilst increasing shelf-life of certain food products, including cheese, even after the package is opened (Shekhon, 2010). Schalkhammer (2012) patented an intelligent 'nanoink' which serves in optimising storage conditions and guaranteeing product quality by indicating the state of the product through monitoring particular analyte levels in cheese. Other areas where nanoparticles can be integrated into active and intelligent purposes, including; scavenging systems (gas and moisture), nano-release systems (antimicrobial and ethanol), temperature control, dirt repellence, antistatic operations, indicators, sensors, biosensors and RFID applications (Smolander and Chaudhry, 2010).

1.3.2.5 Smart Packaging

Smart packaging is the most innovative sector of the packaging spectrum which encompasses aspects of packaging design and the incorporation of mechanical, chemical, electrical and electronic forces, or a combination of these, within the package (Kerry and Butler, 2008). Smart packaging technology is estimated to grow globally at a compound annual growth rate of 4.8% from 2014 to 2020, reaching an estimated €36.5 billion by 2020 (MarketsandMarkets, 2015). Traditional packaging systems, in terms of their provision of basic packaging functions, are not satisfying global market demands. In general, an increase in consumer preference for safety, quality and extended shelf-life are placing greater demands on the performance of

food packaging. In a global sense, geographically distant markets have insufficient transport, storage and distribution operations for most exporters, which heavily compromise product quality and greatly shorten shelf-life due to increased transit times and environmental abuse (excessive movement and handling, fluctuating humidities and temperatures). Therefore, packaging solutions must be created to satisfy the increased demand for superior performance, reduce food waste and troubleshoot supply chain inadequacies to provide a safe and high quality product. 'Smartness' in packaging can be applied to any level of food packaging – primary, secondary, or tertiary to enhance the product experience by actively working with the food product or by communicating food product information. Whilst this branch of packaging is developing rapidly, the commercial uptake is slow. Reasons for this deferment may be due to manufacturers and consumers not perceiving the cost advantages of implementing the technologies or due to perceived concerns surrounding smart packaging legislation. However, whether used directly or indirectly, smart packaging solutions are beneficial to both consumer and manufacturer, as the consumer ultimately receives a safer product and the manufacturer has evidence of the quality and safety associated with their products and processes. As research within the area grows and usage of the technology commercially increases, cost implications are likely to decrease. Despite the field of smart packaging expanding, few active or intelligent applications have been adopted by the dairy industry, and only a limited number have been commercially applied to cheese products.

1.3.2.5.1 Active Packaging

Active packaging changes the condition of the packaged food to extend shelf-life or to improve food safety or sensory properties, while maintaining the quality of the packaged food (De Kruif *et al*., 2002). The active components can help control or regulate certain processes – chemical, physical, microbiological, physiological or infestation, which can improve the nutritive value or organoleptic properties or aid in delaying food deterioration. Active packaging approaches are usually administered via sachet, label, pad, closure, but plastic containers or films are the most prevalent and preferred technique of active packaging as the active agent completely surrounds the food item. Cheese is a suitable product to use in active packaging as the majority of spoilage occurs at the cheese surface. Active packaging systems are usually classed as absorbing, releasing, or other systems.

1. Absorbing systems – These systems remove undesirable compounds from packaging. These packaging systems are designed to scavenge specific constituents to increase shelf-life, improve health or sensory properties and to maintain or improve quality.

Removal of oxygen is important for cheese packaging as the presence of oxygen facilitates microbiological growth and causes oxidation, both of which lead to the production of off-flavours and odours, colour change, nutritional losses and a decrease in food safety and shelf-life. Oxygen absorbers provide an alternative to vacuum packing, modified atmosphere packaging and can be used to compensate for package deficiencies. Existing oxygen scavenging technologies are based on the oxidation of one or more of the following substances: iron powder, ascorbic acid,

photo-sensitive dyes, enzymes (such as glucose oxidase and ethanol oxidase), unsaturated fatty acids (such as oleic, linoleic and linolenic acids), rice extract, or immobilised yeast on a solid substance (Floros *et al*., 1997). Much research has examined the impact of incorporating oxygen absorbers, including commercialised systems, into cheese packaging such as: Oxyban (Scott, 1958), Patent WO/013556 (Aaltonen *et al*., 1991), FreshPax® (Alarcon and Hotchkiss, 1993), Ageless® (Floros *et al*., 1997), ZerO$_2$® (Rooney, 2000), Atco (Panfil-Kuncewicz *et al*., 2006), and ABSO$_2$RB (Gomes *et al*., 2009).

The presence of excess moisture inside the packaging can negatively impact pack appearance and affect the texture and quality of the product, but most critically, it can encourage microbial growth. An effective way of controlling excess water accumulation in a food package that has a high barrier to water vapour is to use a moisture scavenger, such as silica gel, molecular sieves, natural clays calcium oxide, calcium chloride and modified starch, or other moisture-absorbing substances (Ozdemir and Floros, 2004). These scavengers are usually in the form of absorbent sheets or pads. Humidity buffering can also be employed to reduce moisture content within a package. It involves the interception of moisture in the vapour phase by reducing the in-pack relative humidity and thereby the surface-water content of the food (Suppakul *et al*., 2003). It can be achieved by means of one or more humectants between two layers of a plastic film, with the innermost layer being highly permeable to water vapour. Pantaleão *et al*. (2007) demonstrated a successful example of the use of a humidity controller (Humidipak®) on cheese (Saloio) to extend its shelf-life.

Light, and in particular ultraviolet (UV) light, can act as a catalyst in degradation reactions such as oxidation. The use of foil, opaque or orientated plastic-based packaging are possible solutions to restrict induced oxidation. The application of a UV light absorber or blocker can also be used to protect light sensitive foods like cheese. Polymeric hindered amines (HALS), such as Tinuvin 622 and Chimasorb 944, are commonly used in polyolefins as light stabilisers (Lau and Wong, 2000). Kristoffersen *et al*. (1964) used a film containing a UV light screening material (Uvinul D 49) to reduce flavour deterioration in cheese.

Carbon dioxide removers have the potential to be used in the packaging of respiring cheeses. Some cheeses like Emmental and Gouda produce carbon dioxide during ripening. Whilst carbon dioxide is required to achieve, desired texture development and assists in inhibiting organisms, excessive production in a high-gas barrier packaging could cause bloating, or more severely, pack-burst. This bloating or swelling can also give a false indication of an unsafe food and contribute to unnecessary food wastage through discard. Carbon dioxide scavengers could remove a portion of this atmosphere over time to ensure a balanced internal package environment. Fellows *et al*. (2000) describes a one-way valve which permits the release of carbon dioxide from the package without allowing other gases to infiltrate, specifically for use with mould ripened cheese.

Other compounds, like lactose and cholesterol, may be removed from cheese products, using lactase and cholesterol reductase enzymes, respectively. Their removal does not affect shelf-life but enhances the products in terms of composition and nutritional status, especially for those who are lactose intolerant and for those wanting to lower cholesterol intake. Lactose-free cheeses may see a surge in demand due to growth in developing cheese markets, like India and China, which

traditionally have been associated with increased incidences of lactose intolerance within the respective native population. López-de-Dicastillo *et al*. (2011) developed a novel Ethylene Vinyl Alcohol (EVOH) film containing beta-cyclodextrins to remove undesirable food components like cholesterol and aldehydes from milk. The use of off-flavour and odour absorbers, such as aldehyde scavengers, can aid in improving the overall sensory experience of the product and can also prevent food wastage by removing undesirable compounds from the headspace. However it is imperative that the constituents removed are not necessary for flavour development in the cheese and that they are not indicators of spoilage.

2. Releasing systems - These systems actively liberate compounds into the packaging. These emitters add constituents to the packaging or packaging headspace to aid in preservation, maintenance of pack shape, improvement in appearance, odour or flavour, provide protection, advance the quality or add value to the product contained.

Antimicrobial emitting films are the most researched form of smart applications used in cheese packaging to suppress the growth of microorganisms. Antimicrobial agents can be inserted as sachets or pads, flushed within the packaging headspace, coated onto the packaging surface, directly incorporated into the packaging or immobilised onto the packaging by ion or covalent linkages (Appendini and Hotchkiss, 2002). Certain factors must be considered when employing antimicrobial packaging, such as the nature of the food product; the characteristics of the antimicrobial including cost and safety aspects; and the storage condition of the product. The range of preservative agents used in antimicrobial applications varies enormously, with a general trend towards the incorporation of natural substances into the packaging. The

antimicrobials most commonly used in cheese applications include organic acid compounds (including salt derivatives and anhydrides), other fungicides like Imazalil and natamycin, bacteriocins (predominately nisin), enzymes, essential oils, miscellaneous compounds like allyl isothiocyanate, or combinations of these agents (Weng and Hotchkiss, 1992 and 1993; Scannell *et al*., 2000; Var *et al*., 2006; Conceição Gonçalves *et al*., 2009; Hanušová *et al*., 2010; Conte *et al*., 2011; Govaris *et al*., 2011; Hauser and Wunderlich, 2011). The Minimum Inhibition Concentration (MIC) is usually employed for determining the susceptibility of microorganisms to potential antimicrobials as it is a well-established, rapid, inexpensive and reproducible method. Future work is likely to focus on the incorporation of the active agents into, or onto, biodegradable and edible materials, with novel and naturally-derived antimicrobials being of greater interest. Chitosan, a natural polysaccharide, in particular has earned attention due to its biocompatibility, biodegradability, and antimicrobial, filmogenic and metal complexation properties (Martínez-Carnacho *et al*., 2010), with Coma *et al.* (2002, 2003), Duan *et al.* (2007), Di Pierro *et al*. (2011), and Moreira *et al.* (2011) all investigating its use in antimicrobial packaging as a carrier, or as an active component in a film on cheese products. The future will also see increased integration of nanotechnologies within antimicrobial-releasing systems.

Carbon dioxide and ethanol are both known to inhibit bacteria, moulds and yeasts, and both have been used individually as emitters in cheese packaging systems. An atmosphere rich in carbon dioxide may be obtained by using sachets containing iron carbonate, mixtures of ascorbic acid with acid sodium carbonate or a mixture of iron(II) carbonate with metallic halides, which can be used on cheese to prevent package collapse or to inhibit the growth of microbials present (Bilska, 2011). An

ethanol emitter can be incorporated as a film or sachet to retard microbial growth, reduce oxidation and extend shelf-life. Ethanol is usually absorbed or encapsulated in a carrier material that allows the controlled release of ethanol vapour (Day, 2008). Ethicap® is a commercial ethanol emitter which has been used in cheese packaging (Singh *et al*., 2011). High levels of carbon dioxide or ethanol may cause undesirable flavours to be imparted to the product and additional flavour compounds may be added to mask the taint.

Antioxidant releasers can delay or prevent oxidation-led quality deteriorative reactions, and by association, inhibit the formation of off-odours and off-flavours associated with oxidation and therefore, extend shelf-life. Antioxidant emitters may be used as an alternative to oxygen scavengers systems, particularly if anaerobic atmospheres are an issue. Synthetic antioxidants like butylated hydroxyltoluene (BHT) and butylated hydroxylanisol (BHA) have traditionally been used in cheese packaging (Soto-Cantú *et al*., 2008), however, like all other additives the direction of antioxidants is towards naturally-derived systems. López-Gresa *et al*. (2011) found feruloylnoradrenaline (FNA), a derivative from tomato plants, had a much higher antioxidant activity when compared to some established synthetic and natural antioxidants, which encourages further investigation into its use as an antioxidant agent within food packaging.

Colour-, flavour- and odour-releasers and pesticide-emitters are releasing systems which may be applied to cheese packaging in the near future. Colour-releasing films could be used to impart colour to cheese. The films could be used instead of adding the pigment annatto during processing, or where the colour has not been produced as strongly as required, or where colour is adversely affected during distribution. Flavour-emitters could be used to accentuate product flavour in cases where flavour

degradation has occurred during processing. Such an approach would also enable ingredient replacement, combat flavour scalping and to help improve overall product flavour. Flavour emitters may also be used to mask off-odours, but another form of indication should also be used to inform the customer if the food is unsafe to eat. ScentSational Technologies uses an encapsulated aroma release technology to incorporate food grade flavours and fragrances into the packaging, which allows aroma release at different stages, and flavour intensity can be adjusted (www.scentsationaltechnologies.com). Pesticide agents may be applied to the outer packaging layer to ward off pests, insects, or for fungicidal control, which is important for transporting foodstuffs over extended distances, especially products originating from third world countries.

3. Miscellaneous systems

The application of a treatment to the surface of a film can modify the material and impart certain properties, including improved physical, barrier, antimicrobial and other active properties. Modifying the surface includes inserting or altering functional groups on the polymer. Plastics are very convenient for this sort of technology as they can possess active participating elements (López-Rubio *et al*., 2008). An early form of surface treatment includes a patent in which the inner surface of a polyester film is treated by an electrical discharge or by flame treatment to improve adhesion of the film to the cheese (Kane, 1974). This is a temporary adhesion and the cheese pulls away when the package is opened. This anti-stick mechanism is especially useful for individually packaging processed cheese slices as without treatment, cheese has a tendency to stick to the packaging. A more modern approach is via the use of cold-plasma which is a package surface-altering technique,

suitable for heat-sensitive products like cheese. The plasma contains molecules with bactericidal characteristics, which can be imparted to the surface of the packaging. Song *et al*. (2009) evaluated the efficacy of plasma against *Listeria monocytogenes* in sliced cheese, with results demonstrating up to 8-log reductions. Irradiation is commonly used as a surface treatment to introduce functional groups which possess antimicrobial activity. The effects of gamma radiation were shown to prevent *Staphylococcus aureus* growth on cheese sandwiches (Lamb *et al*., 2002). Ozdemir and Sadikoglu (1998) suggested UV-excimer-laser-treatment could be applied to food packaging to provide an antimicrobial characteristic on the inner surface and could replace the usage of ethanol releasing systems with cheese. However, gamma, UV, X-ray and other forms of radiation have had a slow uptake mainly due to consumer suspicion. The application of pulsed-light to packaging surfaces has also proved successful for cheese; Dunn *et al*. (1989) created a patent that utilised intense, short, light pulses on cottage cheese packaging which reduced *Pseudomonas.* Other surface treatments which could potentially be used in cheese packaging to reduce mould growth, include; ozone treatment (Gabriel'yants *et al*., 1980) and ultrasonics (Salo and Wirtanen, 2007).

The combination of two or more smart application systems can be employed to produce a hurdle effect within an individual packaging unit. A patent was created for a packaging material that could be applied to moisture-sensitive products, like soft cheese, which in some cases undergoes a maturing process in the packaging (Marbler and Parmentier, 1999). This packaging material contains at least two functional layers combining moisture and selective permeability. Mexis *et al*. (2011) combined an oxygen absorber with an ethanol emitter in packaging for grated Graviera cheese to achieve a reduction in microbial growth. This active packaging

system had lower microbial counts when compared to both control and MA packaging. A commercialised version, Negamold, has been utilised on cheese as its acts as an oxygen absorber and also generates ethanol vapour (Coma, 2008). DSM has developed Pack-Age® which allows cheese to ripen within a moisture-permeable foil, and is combined with a mould and yeast inhibitor (www.dsm.com). The Biopack project assessed the combination of oxygen scavengers and antimicrobial releasers incorporated into bio-based packaging to significantly extend the shelf-life of cheese (CORDIS, 2005). Other unions that could be used for cheese packaging include moisture absorbing pads used for moisture control linked with antimicrobial releasers or a combination of carbon dioxide or ethanol emitters with oxygen absorbing applications. The partnership of active and intelligent packaging can be used to complement each other's actions. For example, Sängerlaub and Goldhan (2008) evaluated the use of an oxygen scavenger and oxygen indicator employed on a cheese product, conjointly producing a threefold function: absorbing oxygen, monitoring oxygen and appraising package integrity.

Other miscellaneous categories of active packaging which could be applied to cheese include temperature-sensitive films, gas-permeable packaging, self-cleaning rinds, self-cooling or self-insulating containers, and packaging for assisted product heating in microwave ovens. Temperature-sensitive films compensate permeability when temperature fluctuation occurs, frequently during distribution. A predetermined temperature point is designated for the product and when this limit is exceeded, the permeability of the film changes. Although this technology is not applied to cheese, it has the potential to be utilised. Gas-permeable packaging allows gas exchange to occur between the outside environment and inside of the package to ensure an equilibrium that best suits the packaged food product. Coated hydrophobic polymers

containing a high potency hydrosorbent allow the tracking and control of the transfer of water vapour, oxygen and carbon dioxide (Mathlouthi *et al*., 1999). This system works best for cheeses that produce gas on ripening, soft or mould-ripened cheeses. Gerber *et al*. (2011) described the use of an artificial rind prepared from a porous polymer and inoculated with *Penicillium roqueforti*. This smart material provides a self-cleaning ability which replicates the protection that a rind gives to the cheese and can also be permeable to gases if required. For cheese being distributed over long distances, self-cooling or insulating containers may be used to control temperature when the packaging is subjected to temperature abuse from the external environment. The technology is based on endothermic chemical reactions and heat pump technology using water vapour as the heat transfer fluid (Butler, 2008). These technologies compensate for the requirement of constant refrigeration, which is particularly useful when distribution chains are inadequate. A secondary or tertiary thermal management system to ship temperature-sensitive materials may be the most suitable initial introduction to cheese and thermal solutions. Greenbox Systems provide passive thermal solutions engineered as highly efficient shipping containers capable of maintaining narrow temperature ranges for extended durations (www.greenboxsystems.com). The reusable and fully biodegradable system ensures that product arrives at the required temperature to its destination point, regardless of external conditions. As of 2015, Emmi are supplying an innovative microwavable packaging concept to cheese fondue consumers. The All-In-One Fondü contains Swiss cheese within a metal container, which is ready-to-(h)eat, convenient, and recyclable (www.emmifondu.ca).

1.3.2.5.2 Intelligent Packaging

Intelligent packaging systems monitor the condition of the packaged foods to give information about the status of packaged food during transport and storage (De Kruif *et al*., 2002). These systems interact with the food product or its environment, but do nothing to alter the product itself. Intelligent packaging is a form of communication between the food item and the consumer, retailer, or manufacturer. All intelligent forms of packaging could have applications in cheese packaging, however few are available commercially. There are generally two categories of intelligent devices: indicators and data carriers.

1. Indicators – These devices monitor the food product's internal and/or external environment and when necessary provide indication if a certain reaction has occurred. Indicators exhibit a visual response (usually colorimetric) via a label, tag, tablet, or as a layer in a laminate, which may be affixed internally or located externally on the packaging. The following are examples of indicators which have been employed with cheese packaging or that could be potentially applied. These indicators can be used in conjunction with data carriers to determine an estimated shelf-life.

Gas indicators (sometimes known as pack integrity or leakage indicators) usually assess for the presence of oxygen and/or carbon dioxide via the occurrence of a chemical or enzymatic reaction. The gas composition of food package may change depending on the food activity within the pack, permeability of the packaging material, or if any physical damage occurs causing leakage. Knowing the levels of oxygen or carbon dioxide are important to; ensure food quality and safety are

maintained throughout shelf-life, determine if the packaging barrier, seal or the modified atmosphere are functioning as per specification, or to monitor if any gas scavengers present are operating efficiently. Some cheese varieties can produce gas intentionally during maturation; indicators can be used to monitor this progress and indicate when optimum ripening has been attained. Pathogen, microbial growth and freshness indicators determine if safety, quality or freshness have been compromised. They trigger an indication mechanism by detecting the by-products of microbial growth. Physical shock indicators are important in situations where the product is fragile and may be affected through rough handling or transport. Often cheese is subjected to export over large distances and across several countries and it can be difficult to control distribution channels and ensure careful handling by personnel. These indicators can determine if the handling applied is not appropriate and at what point that this occurs during the supply chain. Measures can be applied thereafter to ensure that these critical points can be addressed into the future. Time-temperature indicators confer information concerning the storage conditions of the product. Temperature abuse can affect the product and the package performance leading to nutritional losses, microbial growth and degrade quality which can shorten shelf-life and contribute to food waste. The main mechanisms of time-temperature measuring devices are based on chemical reactions (electrochemical corrosion, enzymatic reactions, polymerisation) and physiochemical properties (melting point, thermal expansion, emissivity, diffusion, solidification temperature, viscoelastic properties) (Estrada-Flores, 2012). An irreversible visual change occurs, when the time/temperature exceeds a predetermined point. This predetermined point depends on the packaged food. Time-temperature indicators are used particularly for foods with long distribution times when temperature fluctuation can occur, or for

temperature-sensitive foods (that are usually stored under frozen or refrigerated conditions like cheese) when an increase in temperature can significantly affect the product. Shellhammer and Singh (1991) used enzyme-based indicators on cottage cheese to show how various temperature conditions that affected cheese quality. Other indicating devices that could be utilised with cheese products include pesticide, antibiotic or allergen indicators.

2. Data Carriers – These systems are useful for applications in cheese packaging due to increased consumer demand for improved convenience, quality and shelf-life. Data carriers transmit information to manufacturers, retailers or consumer, but do not provide the user with a visual response regarding the information they acquire. These devices can be located on the package, as a label, tag or sticker, stationed internally or externally to the primary pack, and can sometimes be incorporated within the packaging material. Carriers can also be integrated with each other or with other smart applications.

Barcodes are the cheapest and most common form of data carriers and are present on the majority of cheese products that are subjected to mass retail. They are used to store product information, track sales and as a reference for stock control. More advanced forms of barcoding are designed to hold additional information, such as product origin, batch number or package weight. The future for barcodes is to make them smaller, but with the capacity to encode more information, for example; cooking instructions, nutritional information, manufacturers' website, social information pertaining to product manufacture, special offers and competitions and the provision of all information in multiple languages. Another intelligent technology development which is similar to barcodes, is based on Near Field

Communication (NFC) technology. An information code or quick response (QR) code is placed on the package, like a barcode, and using a smartphone equipped with an NFC reader, the consumer can download product details (as indicated above). This information can then be conveyed through a speech-based item identification system which allows visually impaired, blind and elderly people to identify food items (Harjumaa, 2012).

Sensors or biosensors are data carriers which can be used to detect food constituents, additives, and contaminants (e.g. pesticides, antibiotics or hormones), by measuring components present, products from reactions (microbial growth, chemical, enzymatic) or measure matter levels within the headspace and store or transmit this information. The variable is sensed and this is then transformed into a quantifiable signal, which can then be recorded by an external device. Unlike indicators, no visual indication is presented via the signal, but the results are recorded and monitored by the manufacturer to provide important information regarding; pack performance, packaging process, and storage conditions. Woodward *et al*. (2005) stated that Yellow Springs Instruments biosensor has been used to detect the presence of lactose in cheese. O' Mahony *et al*. (2006) used an optical oxygen analyser to non-destructively assess residual oxygen levels in cheddar cheese packs using sensors. High levels of oxygen were detected as a consequence of losing packaging containment, which displayed the commercial significance of this technology. Environmental conditions (temperature, light, humidity) surrounding packaging sliced Emmental cheese were monitored using a multi-sensor circuit (Grassi *et al.*, 2012). The sensors demonstrated that quality and safety in storage and delivery could be assured through the employment of this smart system. The future success of increased usage and commercial uptake depends on making the presence

of the sensor more discreet or ‘invisible’ and to further demonstrate the relevance of sensor technology to the industry.

Radio Frequency Identification (RFID) tags are used as an identification tool using wireless microchips, which employ radio frequencies to track the product and pass detected information to the manufacturer or retailer. In 2015, the value of the entire RFID market was worth €9.3 billion, and it is forecast to rise to an estimated €12.2 billion by 2020 (Das and Harrop, 2015). RIFD tags can confirm when a product has been taken off the shelf and purchased, or can help track stolen items and help identify and authenticate them. Advantages of RFID tags also include a reduction in food waste and more efficient assistance during product recall. RFID’s can be thermo-sensitive, which is important for temperature-sensitive products like cheese. Cheese can also be tagged to optimise the manufacturing process, specifically the ripening practice, thereby determining the ripening time and holding conditions for optimised product quality. RFID technology could prove to be invaluable as a technology to guarantee product authenthicity and in the process of tracking and tracing expensive products during cold-chain distribution. Regattieri *et al*. (2007) and Barge *et al*. (2014) both have developed a traceability systems for hard cheese which was based on RFID which allows for tracking of the entire supply chain and provided consumers with the ability to confirm the origin of the cheese. Another application is ‘Smart Shelf’ which is an array of RFID antennae that can identify a product’s location. It has been used on processed cheese to track expiry dates; however, widespread implementation has met obstacles such as the cost and the issue of breach of consumer privacy (Bornhovd *et al*., 2004).

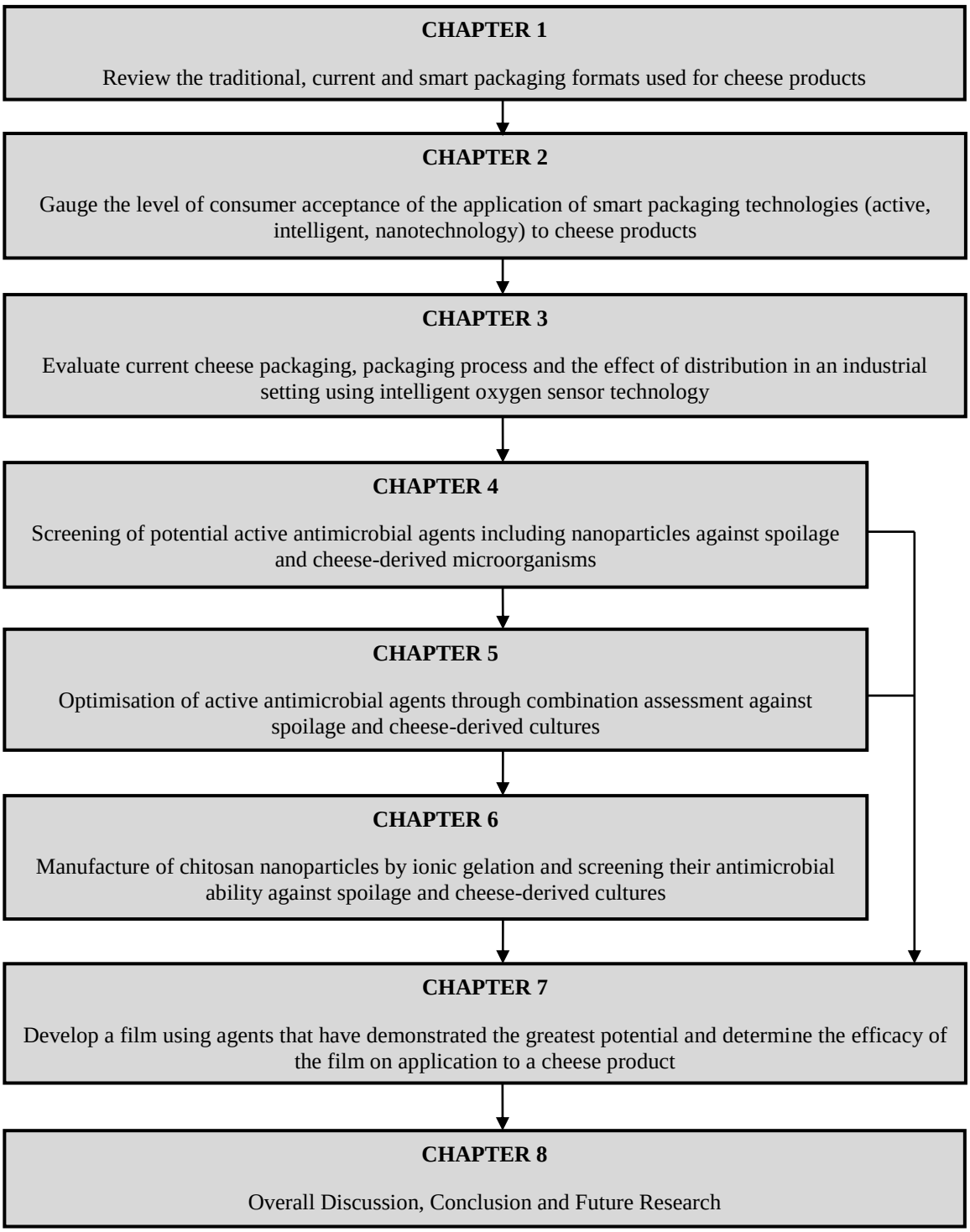
1.4 CONCLUSION

As long as people continue to eat cheese, there will be a need for cheese packaging. The future of cheese packaging will be driven by inaugurating markets and by increased demands from established markets. Manufacturers will need to seek to achieve global exportation by pursuing markets in terms of growth and value, regardless of proximity or lack of adequate distribution channels. For the mature cheese consumers, product expectations will increase to include; more innovation, efforts for healthier, natural products, improvement of function, convenience and safety, whilst minimising cost and reducing wastage by being more environmentally conscious. These increased demands and expectations are not conducive to the function of traditional packaging. Traditional cheese packaging allows cheese products to be protected and delivered over short distances and are therefore unchallenged by domestic distribution. The usage of advanced packaging technologies will expand exponentially in the coming years due to the shortcomings of traditional packaging, changing consumer preference, the development of new and geographically challenging markets, demands for extended shelf-life and enhanced traceability, concerns regarding health, safety and food waste, and the drive for innovative and novel products. The future of smart packaging will focus on extending functions, but with greater invisibility and lower costs. The scope at which smart packaging will be applied to cheese products depends on future research and development in this area as it currently stands underdeveloped. It is important to invest in the packaging of cheese as it not only provides solutions and makes cheese export friendly and more valuable, but it can be seen as a marketing advantage. For innovative packaging to have an impact, it must become commercially viable. This is

achievable through further research, development, and increased usage by cheese producers and packaging manufacturers alike.

Thesis Overview Schematic

Thesis Objectives: Utilising smart packaging technologies, assess the function of current packaging using intelligent technology, evaluate and optimise potential active agents to use in active cheese packaging and develop an active package for use with cheese products.



EXPERIMENTAL CHAPTERS

CHAPTER 2

Consumer attitudes towards the application of smart packaging technologies to cheese products

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ABSTRACT

Higher expectations from retailers and consumers in terms of quality and shelf-life and the greater demand internationally for perishable products like cheese which results in a more challenging distribution, have led to the increasing application of smart packaging technologies to food products. However, one the greatest obstacles which prevents the widespread implementation of such necessary technologies is public resistance. It is therefore imperative to gauge consumer acceptance and decipher what are the specific concerns of the consumer and derive a solution to alleviate these concerns. The survey conducted set out to explore consumer (n=814) knowledge and attitudes pertaining to primary cheese packaging format, cheese shelf-life expectation and advanced packaging technologies such as smart packaging, active packaging, intelligent packaging and nanotechnology. Subsequently, participants were presented with descriptions of these packaging technologies and acceptance evaluated. Willingness to pay more for the extension of shelf-life using these technologies was also assessed. Nanotechnology derived the highest level of awareness, with the other technologies receiving much lower levels of recognition. Consumer acceptance of smart packaging technologies varied depending on technology type and cheese application. Willingness to pay more for products containing these technologies was deemed unacceptable; however, willingness increased after participants received information about the value of using such technologies. Results indicate that provided product recipients are sufficiently educated and the cheese application is warranted, the future is optimistic for the employment of smart technologies to cheese products.

2.1 INTRODUCTION

The role that food packaging plays in product preservation is more prominent than ever. Food products now move along a much more extended distribution supply line, which becomes even more challenging when a chilled supply chain must be maintained. Greater movement over greater distances, coupled with increased handling and fluctuating environmental conditions, means that packaging systems must be more robust in order to deliver levels of safety and product shelf-life that are acceptable to those receiving the goods at the user end of the distribution chain. Additionally, beyond the food industry's need to remain competitive globally, the role of conventionally-employed packaging materials and formats is being challenged further. Rapid shifts in economic stability and changes in population demographics and lifestyles have led to increased demands of added value from food packaging, with obvious benefits for the consumer, particularly in terms of improvements to food quality and safety like enhancement food composition or nutrition, extension of shelf-life, bettering of convenience and security aspects, whilst keeping on trend with packaging technology innovations (Kerry and Butler, 2008). Successful consumer-centred and challenge-focussed packaging can benefit a company by creating a competitive advantage, increasing customer satisfaction and boosting sales volume (Ryynänen and Rusto, 2014). However, packaging activities are often perceived as an unnecessary cost rather than an investment (Simms and Trott, 2010), when in reality, poorly applied, chosen or designed packaging can have a negative impact on cost or performance or both.

Smart packaging technologies are steadily becoming the solution to satisfying the response to consumer demands and industry trends. Within the smart technologies market, food packaging represents a very small fraction, which is almost totally

concentrated in Japan, (Dainelli *et al*., 2008). Smart packaging systems can generate an enhanced product by utilizing non-traditional packaging functions to provide safer and securer, more nutritious or appealing food products, whilst being environmentally friendly. They can also contribute informatively, yielding improved logistical efficiency and optimized product recall. Despite the numerous benefits bestowed by smart packaging, there are several barriers to full-scale adoption and application of such technologies to food products, including: complete scientific knowledge pertaining to the operation and stability of systems; full and complete contact material compliance with food and beverage products; environmental impact and implications of using such technologies on recycling activities; unclear regulatory guidelines, and critically, acceptance by retailers and consumers alike (Coles and Frewer, 2013; Frewer *et al*., 2011). Retailer and consumer attitudes towards food technologies are critical as they can ultimately lead to market success or widespread failure. However, consumers can be too conservative when it comes to accepting innovative concepts (Heiskanen *et al*., 2007). The success of an innovation also depends on the product to which the technology is applied and the technology in question (Murray and Delahunty, 2000). Cheese, like many food product types, is suitably disposed to the application of these packaging technologies. This is because, firstly, it is widely consumed and consumption is growing globally (Sheehan, 2013) and secondly, due to its perishable nature, particularly when opened, spoilage occurs mainly at the product surface. The opinion of the public towards a new technology can be heterogeneous and attitudes may vary dependent on the characteristics of the technology, the level of technology neophobia or consumer's associations with other technologies (Frewer *et al*., 2011). Therefore different technologies can provoke different responses. In order to avoid alienating the consumer and to ensure an

opportunity for adoption success, consumer reactions towards these technologies and potential obstacles should be considered prior to introduction.

Research in the area of advanced packaging technologies and cheese is limited. Murray and Delahunty (2000) observed consumer preference for packaging attributes (shape, aesthetics, performance, presentation) of cheddar cheese, and Bech-Larsen (1996) studied attitudes towards the importance of environmental and functional characteristics of packaging of cheese spread. However, neither explored the opinions of new packaging technologies. Almli *et al*. (2011) determined consumer acceptance of different innovations, including packaging, on cheese. The packaging innovations evaluated were singular examples of convenience- (odour containment) and market- based packages. Most recently, Pilone *et al*. (2015) concentrated on consumer acceptance of environmental and shelf-life extension innovations on Italian cheese, but did not discuss the specific technologies employed to achieve these functions. This investigation was carried out to determine the importance of packaging and packaging attributes of cheese products to the consumer, evaluate opinions on the shelf-life offered by current cheese packaging formats and assess knowledge and attitudes towards the incorporation of novel packaging technologies within these formats in order to further extend product shelf-life or communicate information with respect to product quality, with a particular focus on willingness to purchase if the price was increased.

2.2 MATERIALS AND METHODS

2.2.1 Research questions and survey distribution

The survey was composed of 18 questions (available in appendix). Prior to beginning the questionnaire, participants were informed that its purpose was to 'evaluate consumer attitudes towards cheese packaging, shelf-life of retail cheese products and to assess knowledge and opinions of the incorporation of additional packaging technologies within conventional cheese packaging formats'. It was also specified in this initial introduction, that ideally, the survey participant should be a consumer of cheese. In order to recruit participants, the survey was distributed online through use of the university's survey mailing lists and social media websites.

The start of the survey, pages 2 and 3, contained questions (1 to 7) regarding some basic background information required for each respondent, such as; age, gender, nationality and education level. Education level responses were as follows; Primary school or Secondary school (PS), Post leaving course, Further education and training course or an Apprenticeship (PFA), Third level certificate, Diploma or University degree (TDU), and, Masters degree, Postgraduate diploma, Doctoral degree or Higher doctorate (MPDH). Data were also collected from each respondent with respect to cheese consumption, type of cheese product consumed (soft, hard or both), and varieties purchased most frequently. Questions 8 to 18 - Page 4 assessed consumer views with respect to the manner in which cheese is packaged currently and which packaging attributes were considered important to the consumer. The attributes evaluated, included; containment, appearance, provision of information, convenience, shelf-life, presence of quality marks, tamper evidence features and environmental impact. On page 5 of the survey, participants were asked to estimate

what they thought the shelf-life of cheese to be, their satisfaction with current cheese shelf-life, and the point at which they ceased consuming a cheese product. Page 6 of the survey determined consumer opinion on the application of safe technologies to further extend cheese storage capabilities and their willingness to pay more for this enhancement. Page 7 of the survey asked participants about their knowledge of the following packaging or related terms; Smart packaging, Active packaging, Intelligent packaging and Nanotechnology. If respondents had heard of the term they were also asked to comment on the circumstance in which they had been introduced to the term and whether it was in a positive or negative context. The final page of the survey (page 8) provided the participants with a description of each of the terms, which were presented as follows;

Smart Packaging – A package that provides the consumer with an extra function beyond the basic purpose of the package (protection, containment and communication). The extra function is usually mechanical, chemical, electrical or electronic.

Active Packaging – This is a form of smart packaging. An active package contains constituents incorporated into the packaging material or within the packaging container that deliberately alters the condition of the package to either enhance sensorial properties, maintain or improve quality, or to extend the shelf-life of the packaged product.

Intelligent Packaging – This is a form of smart packaging. An intelligent package contains a device, positioned internally or externally to the package, which can monitor the condition of the product, package or packaging environment. The device

can provide information on these aspects, but does not alter the condition of the package or product.

Nanotechnology – This is the use of materials on a nanometre scale, between 1 nm and 100 nm in size (1 nm = 1 millionth of a millimetre). Nanoparticles can expand the performance range of existing packaging materials. Particles at this size exhibit novel properties such as improved activity, mechanical, thermal and barrier function.

Acceptance was determined by asking consumers whether they would purchase a cheese product whose packaging contained one or more of these technologies. The final question repeated the query regarding paying more for the use of such technologies in retail packs of cheese products. Questions 8 to 18, with the exception of Q16, and their respective responses are presented in Tables 2, 3, 4 and 6.

2.2.2 Statistical evaluation

Completed questionnaires were coded into a Microsoft® Excel worksheet and transferred into SPSS Statistics 20 (IBM, Armonk, NY, USA) to perform statistical analysis. Data were summarized as frequencies for each question and presented in contingency tables. Significance was determined using Chi-square analysis, and where these statistical differences existed, were identified using Chi-square post-hoc tests (Beasley and Schumacker, 1995). A significance level of $P \leq 0.05$ was set and this was adjusted to control the type I error rate. The adjusted P value = 0.05/Number of analyses performed. A paired t-test was employed to establish if there was a significant difference ($P \leq 0.05$) between responses for questions 14 and 18.

2.3 RESULTS AND DISCUSSION

2.3.1 Participant demographic

A total of 814 complete responses were collected from the survey. Respondent demographic characteristics are presented in Table 1. Respondents were mostly aged between 18 to 34 and the majority of respondents (39.43%) had completed a PS level of education, both of which are unsurprising since the survey was distributed via university channels. This can also explain the increased number of female responses, 67.08% females compared to 32.92% males, as the IUA (2013) reported that in the academic year 2009/2010, more females than males were in enrolled in Irish universities. Additionally it has been noted the gender balance in voluntary food related surveys is often skewed, with females over representing (Van Boxstael *et al*., 2014). Despite being circulated within an Irish university environment, 33 nationalities responded, with Ireland, the United Kingdom (inclusive of England, Scotland, Wales and Northern Ireland), Germany, the United States, Malaysia and Canada, contributing the bulk of responses. Most participants consumed cheese regularly (daily or weekly), with more than half (64%) eating both hard and soft cheese. Respondents were also asked to provide the names of the varieties of cheese they consumed most often. In total, 70 cheese varieties were mentioned, with the most popular cheese types being (those noted over 50 times); Cheddar, Mozzarella, Parmigiano-Reggiano/Parmesan, Cream cheese, Brie, Blue cheese (Stilton, Cashel blue or Gorgonzola), Goats cheese, Feta, Edam, Swiss cheese/Emmental, Gouda and Cottage cheese.

Table 1 – Respondent demographic

	n	%
Age		
18 to 24	538	66.09
25 to 34	178	21.87
35 to 44	54	6.63
45 to 54	25	3.07
55 to 65	11	1.35
65 or older	8	0.98
Gender		
Female	546	67.08
Male	268	32.92
Education level completed		
Primary school or Secondary school (PS)	321	39.43
Post leaving course, Further education and training course or an Apprenticeship (PFA)	89	10.93
Third level certificate, Diploma or University degree (TDU)	276	33.91
Masters degree, Postgraduate diploma, Doctoral degree or Higher doctorate (MPDH)	128	15.72
Estimation of cheese consumption		
Daily	356	43.73
Weekly	412	50.61
Monthly	31	3.81
Rarely	15	1.84
What type of cheese do you consume:		
Soft cheese	62	7.62
Hard cheese	231	28.38
Both soft and hard cheese	521	64

2.3.2 Packaging importance

As observed in Table 2, more individuals, overall, considered the manner in which cheese is packaged to be important to them (59.2%). All divisions of each group (age, gender, education) had a higher proportion of 'Yes' respondents, with the exception of the over 65 participants, or, those possessing a PFA education, both of which received a higher 'No' response. Although no significant differences were found within age, gender or education, a number of patterns were determined. With the exception of the over 65's, the importance of packaging increased as participant

Table 2 - Importance of packaging and packaging attributes.

	Age (%)						*P value*	Gender (%)		*P value*	Education (%)				*P value*	
	18 to 24	25 to 34	35 to 44	45 to 54	55 to 64	65 or older		Female	Male		PS	PFA	TDU	MPDH		Total (%)
Q8 Is the manner in which the cheese is packaged important to you?							0.176			0.310					0.072	
Yes	57.10	62.40	63.00	72.00	81.80	37.50		60.4	56.7		57.9	48.3	63.8	60.2		59.2
No	42.90	37.60	37.00	28.00	18.20	62.50		39.6	43.3		42.1	51.7	36.2	39.8		40.8
Q9 Score each of the following packaging features in terms of importance to you.																
Product is contained and properly sealed.							0.000			0.019					0.051	
Very Important	89.2	86.0	81.5	80.0	63.6	$25.0^{0.000}$		$89.0^{0.006}$	$82.1^{0.006}$		$91.3^{0.002}$	85.4	85.1	79.7		86.7
Somewhat Important	8.4	10.7	13.0	16.0	27.3	25.0		$7.9^{0.008}$	$13.8^{0.008}$		6.5	11.2	10.5	15.6		9.8
Not Important	2.4	3.4	5.6	4.0	9.1	$50.0^{0.000}$		3.1	4.1		2.2	3.4	4.3	4.7		3.4
Pack shape.							0.740			0.696					0.428	
Very Important	5.6	6.7	7.4	8.0	9.1	12.5		5.9	6.7		4.0	10.1	6.9	7.0		6.1
Somewhat Important	36.8	37.1	31.5	20.0	45.5	12.5		35.2	37.3		36.1	33.7	37.3	33.6		35.9
Not Important	57.6	56.2	61.1	72.0	45.5	75.0		59.0	56.0		59.8	56.2	55.8	59.4		58.0
Degree of decoration or appearance.							0.454			0.104					0.390	
Very Important	8.2	12.4	3.7	8.0	9.1	12.5		8.1	10.4		7.2	5.6	11.6	9.4		8.8
Somewhat Important	44.4	42.1	42.6	32.0	45.5	12.5		45.6	38.1		44.5	39.3	43.1	42.2		43.1
Not Important	47.4	45.5	53.7	60.0	45.5	75.0		46.3	51.5		48.3	55.1	45.3	48.4		48.0
Provision of adequate information on the label or printed on the package.							0.180			0.000					0.758	
Very Important	58.0	62.9	53.7	44.0	63.6	37.5		$63.0^{0.000}$	$48.5^{0.000}$		57.6	59.6	60.9	53.1		58.2
Somewhat Important	32.3	25.3	38.9	40.0	27.3	25.0		28.4	37.3		32.4	29.2	28.3	36.7		31.3
Not Important	9.7	11.8	7.4	16.0	9.1	37.5		8.6	14.2		10.0	11.2	10.9	10.2		10.4
Convenience features such as easy opening or resealability.							0.024			0.114					0.396	
Very Important	47.0	34.8	38.9	52.0	36.4	37.5		46.0	39.2		43.6	44.9	44.6	41.4		43.7
Somewhat Important	41.6	50.0	40.7	24.0	54.5	25.0		41.9	44.8		46.1	41.6	39.1	43.8		42.9
Not Important	11.3	15.2	20.4	24.0	9.1	37.5		12.1	16.0		10.3	13.5	16.3	14.8		13.4
Storage, stability and shelf-life of the packaged product.							0.001			0.006					0.077	
Very Important	$63.4^{0.003}$	53.4	40.7	68.0	63.6	50.0		$63.6^{0.001}$	$51.9^{0.001}$		60.7	65.2	58.0	57.0		59.7
Somewhat Important	31.4	37.6	50.0	24.0	27.3	12.5		$30.2^{0.004}$	$40.3^{0.004}$		35.5	29.2	31.9	35.2		33.5
Not Important	5.2	9.0	9.3	8.0	9.1	$37.5^{0.001}$		6.2	7.8		3.7	5.6	10.1	7.8		6.8
Use of quality marks, symbols and icons, e.g. guaranteeing traceability or origin.							0.009			0.121					0.705	
Very Important	36.1	38.8	42.6	24.0	54.5	25.0		38.8	32.8		34.9	42.7	37.7	35.9		36.9
Somewhat Important	46.3	44.9	42.6	72.0	45.5	12.5		45.8	47.0		45.8	44.9	46.7	46.9		46.2
Not Important	17.7	16.3	14.8	4.0	0.0	$62.5^{0.001}$		15.4	20.1		19.3	12.4	15.6	17.2		17.0
Presence of tamper evidence features or tamper-proof seals and closures.							0.391			0.003					0.152	
Very Important	41.8	42.1	48.1	44.0	54.5	25.0		$46.5^{0.001}$	$34.0^{0.001}$		41.1	37.1	48.2	36.7		42.4
Somewhat Important	40.0	38.2	29.6	32.0	45.5	25.0		35.9	44.0		40.5	46.1	32.2	42.2		38.6
Not Important	18.2	19.7	22.2	24.0	0.0	50.0		17.6	22.0		18.4	16.9	19.6	21.1		19.0
Environmentally friendly aspects.							0.021			0.356					0.756	
Very Important	23.4	29.2	31.5	32.0	36.4	12.5		26.9	22.8		26.2	29.2	25.0	22.7		25.6
Somewhat Important	48.3	52.8	38.9	52.0	63.6	25.0		48.5	49.3		47.0	48.3	51.4	47.7		48.8
Not Important	28.3	18.0	29.6	16.0	0.0	62.5		24.5	28.0		26.8	22.5	23.6	29.7		25.7

Education level abbreviations - Primary school or Secondary school (PS), Post leaving course, Further education and training course or an Apprenticeship (PFA), Third level certificate, Diploma or University degree (TDU), Masters degree, Postgraduate diploma, Doctoral degree or Higher Doctorate (MPDH).
P value represents level of significance within a group. Superscript values indicate where this significance lies.

age increased; females considered the way in which cheese was packaged to be more important than males, and the greater the level of education received by individuals (MPDH and TDU) who participated in the survey, the greater the importance attached to packaging usage. These groupings of individuals most likely have a greater awareness of the role played by food packaging due their increased experience, exposure and education, respectively.

Packaging attributes are central to the consumer selection process (Murray and Delahunty, 1999). The packaging features that respondents held in greatest importance were; proper containment of the product, stable shelf-life, and the provision of information on the package, respectively. Containment and seal-

integrity was rated the most important attribute overall and this makes sense as it is a basic and a fundamental function of packaging. Bech-Larsen (1996) also determined the functional characteristic of sealing to be important for the packaging of cheese spread. In contrast with our findings, the importance of shelf-life as an attribute in cheese packaging was determined to be less relevant in a study conducted by Pilone *et al*. (2015) and was rated second last overall from a listing of packaging terms employed. Peters-Texeira and Badrie (2005) also found the presence of information influenced consumer choice on purchasing more than any other packaging feature. In our study, pack shape, appearance of the package and environmental concerns were considered to be the least important packaging attributes. Pack shape did not rate highly either when Gelici-Zeko *et al*. (2013) studied responses to dairy product packaging, however, package appearance was considered the most important feature, which is in contrast to our findings. Despite environmental concerns being very much to the packaging forefront in recent years, respondents in this study marked it as an aspect of packaging that was of lesser importance. It has been suggested that environmental characteristics are only paramount to consumers specifically interested in environmental issues (Bech-Larsen, 1996). So while these aspects are important to a select grouping of consumers; they represent only a small portion of the entire consumer population.

Within age, the oldest grouping (over 65's) showed the greatest discrepancy, with significant differences being determined within containment (very important – $P \leq 0.000$, not important – $P \leq 0.000$), shelf-life (not important – $P \leq 0.001$) and quality marks (not important – $P \leq 0.001$) features. There was a major contrast between what older and younger respondents felt was important in terms of packaging features. Older respondents did not attach much importance to issues such as; containment,

shelf-life or quality marks. This may point to a different set of criteria being used by this consumer grouping to determine acceptable product quality. For example, quality assurance marks on packaging is a relatively new concept which older people may not be interested in or associate any product value with. Females held most attributes at a greater importance, except for pack shape and appearance, which males considered more important. Women are generally recognized as caregivers and consequently, are usually more concerned about food health and safety (Chen *et al*., 2013). Therefore, it is unsurprising that females considered containment, information, shelf-life and tamper evidence features to be very important; findings which were significantly different from those determined for males ($P \leq 0.006$, $P \leq 0.000$, $P \leq 0.001$, and $P \leq 0.001$, respectively). This demonstrates the necessity for females to ensure the food they purchase is safe and secure, not only for personal consumption, but for those they provide for also. Education groups were generally in agreement, with exceptions being noted for containment and sealing features. A PS education considered this characteristic to be very important, which was significantly different ($P \leq 0.002$) from the other educational respondent groups. Despite the importance of containment and sealing decreasing as education level increased, it is still the highest scoring attribute overall, most likely because this feature is not seen as a functional property, but more like a basic requirement of packaging.

2.3.3 Opinions on cheese shelf-life

The majority of respondents concluded that they expect cheese to store mostly for weeks (61.1%), followed by days (33.7%) and then months (5.3%) (Table 3). When

Table 3 - Cheese shelf-life

	Age (%)						*P value*	Gender (%)		*P value*	Education (%)				*P value*	Cheese consumption (%)				*P value*	Type of cheese consumed (%)			*P value*	
	18 to 24	25 to 34	35 to 44	45 to 54	55 to 64	65 or older		Female	Male		PS	PFA	TDU	MPDH		Daily	Weekly	Monthly	Rarely		Soft	Hard	Both		Total (%)
Q10 When you purchase cheese products, how long do you expect the product to store for?							0.047			0.464					0.505					0.584				0.005	
Days	36.4	28.1	24.1	40.0	36.4	12.5		32.2	36.6		37.7	30.3	29.3	35.2		34.6	33.0	29.0	40.0		37.1	$24.7^{0.001}$	$29.0^{0.004}$		33.7
Weeks	58.4	68.0	70.4	48.0	63.6	62.5		62.5	58.2		57.6	64.0	64.9	59.4		60.1	62.4	58.1	53.3		53.2	$69.3^{0.003}$	58.1		61.1
Months	5.2	3.9	5.6	12.0	0.0	25.0		5.3	5.2		4.7	5.6	5.8	5.5		5.3	4.6	12.9	6.7		9.7	6.1	12.9		5.3
Q11 Are you satisfied with current cheese shelf-life?							0.924			0.799					0.842					0.617				0.913	
Yes	86.2	84.8	81.5	88.0	90.9	87.5		85.5	86.2		85.4	87.6	84.8	87.5		87.4	84.7	80.6	86.7		87.4	84.7	80.6		85.7
No	13.8	15.2	18.5	12.0	9.1	12.5		14.5	13.8		14.6	12.4	15.2	12.5		12.6	15.3	19.4	13.3		12.6	15.3	19.4		14.3
Q12 When do you stop consuming a cheese product following purchase?							0.065			0.213					0.138					0.000				0.035	
Sell by date	1.9	1.1	3.7	4.0	0.0	0.0		1.3	3.0		2.2	2.2	1.4	1.6		1.4	2.4	0.0	0.0		1.4	2.4	0.0		1.8
Best before date	18.4	14.0	7.4	4.0	0.0	0.0		17.2	13.1		19.6	11.2	13.8	14.1		14.6	14.1	$35.5^{0.002}$	$53.3^{0.000}$		14.6	14.1	35.5		15.8
Use by date	18.0	11.8	11.1	12.0	27.3	0.0		15.0	17.9		19.3	13.5	14.5	12.5		12.4	19.4	16.1	6.7		12.4	19.4	16.1		16.0
After the expiry date	4.8	3.4	1.9	8.0	9.1	0.0		4.6	4.1		5.3	5.6	3.6	3.1		4.2	4.6	3.2	6.7		4.2	4.6	3.2		4.4
Cheese acceptability based on sensory characteristics	$56.9^{0.000}$	69.7	75.9	72.0	63.6	100.0		61.9	61.9		$53.6^{0.000}$	67.4	66.7	68.8		67.4	59.5	45.2	33.3		$67.4^{0.001}$	59.5	45.2		61.9

Education level abbreviations - Primary school or Secondary school (PS), Post leaving course, Further education and training course or an Apprenticeship (PFA), Third level certificate, Diploma or University degree (TDU), Masters degree, Postgraduate diploma, Doctoral degree or Higher Doctorate (MPDH).

P value represents level of significance within a group. Superscript values indicate where this significance lies.

considering that this estimation considers both soft and hard cheese, this result is to be expected. Concurrently, no significant differences were determined between days, weeks and months, within the groups of age, gender, education and frequency of cheese consumption, however, within type of cheese consumed, significant differences were observed ($P \leq 0.01$). Soft cheese had the highest score (37.1%) for a storage time of days, which is as anticipated due to its perishable nature. Hard cheese was found to be significantly different ($P \leq 0.003$) from soft cheese and both cheeses at a storage of weeks, which again is as expected due to its lower moisture levels and longer shelf-life.

In terms of consumer satisfaction with current cheese shelf-life, there was complete agreement across all groups assessed, with the majority confirming they were sufficiently happy with retail cheese shelf-life at present (85.7%). The most common response, and most probable reason, for such a high level of consumer satisfaction was due to the frequent consumption of cheese. Therefore, the fact that cheese is consumed so rapidly within the household means that cheese products do not have time to spoil. Consumers also stated that cheese is a perishable dairy product and some consumers just accepted that cheese naturally goes off at a certain point. Consequently, consumers holding such a view would probably never see the point of trying to extend product shelf-life in any case. Additionally, but not surprisingly, cheese type was a large determinant in shelf-life satisfaction. Consumers who do worry about shelf-life purposely sought out cheese with the longest shelf-life in the supermarket. Cheese variety was acknowledged as having an impact on shelf-life, with processed and hard cheeses having longer shelf-lives, and soft cheeses recognized as having a shorter shelf-life. This was generally accepted as a trade-off for soft cheese, but comments were made that it would be useful to extend the shelf-

life of soft cheeses. Cheese format was also found to be associated with shelf-life, with grated and sliced cheeses being noted for deteriorating faster. Unlike whole forms, these product formats are harder to salvage if spoiled, and therefore, consumers were often dissatisfied with grated and sliced packaging formats. Many respondents inferred that packaging and storage often affected the storage stability of their cheese, with some experiencing spoilage of cheese prior to even opening the cheese pack. The majority found spoilage occurred once the package was opened, with consumers stating that the direction on the package, to consume the cheese within three days of opening was unrealistic. This spoilage often occurred prior to the expiration of shelf-life dates printed on the package and despite following rigorous temperature control, with consumers finding this unpredictability annoying. Consumers commented that the shelf-life of cheese could be extended with adequate packaging. Packaging was found to be insufficient by many respondents and this was indicated by packs easily propagating tears on opening and not being resealable. Resealability is used to reduce exposure to air and is often employed in cheese packaging; however consumers stated that this feature cannot prevent the trapping of air upon opening and consequently, does not accommodate the maintenance of product shelf-life. Many respondents commented that they extended the shelf-life of the cheese they purchased by employing additional packaging at home (airtight container, cling film, wax paper, resealable bags) once the cheese products were initially opened. It is also worth noting that respondents from other countries like Canada and Australia mentioned that the shelf-life of cheese sold in Ireland was considerably shorter than cheese products retailing in their native countries. They also commented that product deterioration appeared to occur much more quickly with Irish products. All of this may be due to compositional differences between

cheese products or may be due to differences in packaging materials and systems employed to package cheese products in different parts of the globe.

It was quite apparent that respondents to this survey primarily depended on their sensory assessment abilities (61.9%) rather than using any perishability date proposed on the package when making judgment on the consumption suitability of a cheese product. It is evident from this survey that younger participants (aged 18 to 24 years) are more dependent on estimated shelf dates than are older participants. This difference in attitude was significant on scoring for the response 'cheese acceptability based on sensory characteristics'; where the 18 to 24 age category had the lowest score (56.9%) and was significantly different ($P \leq 0.000$) from all other age ranges. There were no significant gender differences with respect to selection criteria used for packaged cheese. These results are in agreement with Van Boxstael *et al*. (2014), who also established edibility was judged primarily using a combination of smelling and visual inspection, and that older people were more willing to eat expired cheese, with no significant differences determined for gender. Those possessing a PS education seemed more reliant ($P<0.000$) on shelf-life dates than those who had achieved further education. It could be derived that a portion of those with a PS education may not have finished their education as yet and are presumably of a younger age, which aligns with the result realized within the age 18 to 24 years age category. Unsurprisingly, infrequent consumers (those who consume cheese monthly to rarely), were more dependent on dates, particularly with respect to the best before date, both of which were significantly different ($P \leq 0.002$ and $P \leq 0.000$, respectively). It could be assumed that because this consumer grouping consume cheese less regularly and consequently, may not be as familiar with the signs of spoilage, thereby making decisions based on sensory characteristics less

likely. Respondents who consumed only soft cheese were most dependent on sensory evaluation, which was significantly different ($P \leq 0.001$) from hard cheese and both cheese types. This finding is unusual as it was assumed that due to the rapid nature of soft cheese spoilage, coupled with the fact that visible signs of spoilage are not always apparent with this type of cheese, that shelf-life dates would be important cues in reaching judgment on consumption. These overall results suggest that consumers assess the consumption quality of cheese in a very flexible manner, and that quite possibly, dates relating to shelf-life on cheese packages are largely redundant.

2.3.4 Knowledge of quality and shelf-life enhancing technologies

Nearly two-thirds of respondents (66.0%) were found to be in favour of the use of safe technologies with the specific purpose of informing on product quality or extending product shelf-life (Table 4). Within age categories, 35 to 44 year olds ($P \leq 0.000$) and over 65's were less likely to welcome the use of new technologies in cheese packs. Male participants were more willing to accept ($P \leq 0.004$) the use of new technologies in the extension of cheese shelf-life compared to females. There were no significant differences determined between participants possessing different educational levels. These findings are consistent with Brook Lyndhurst Ltd. (2009) who found older people and women more concerned, less-positive and more likely to perceive fewer benefits associated with smart packaging technologies. Additionally, the company also reported that no evident patterns emerged with regard to education levels, which again agrees with findings reported in our work. It was apparent that the type of technology used would be critical to consumer acceptance,

Table 4 - Knowledge of technologies

	Age (%)						*P*	Gender (%)		*P*	Education (%)[1]				*P*	
	18 to 24	25 to 34	35 to 44	45 to 54	55 to 64	65 or older		Female	Male		PS	PFA	TDU	MPDH		Total (%)
Q11 If there were safe technologies that could be used to extend cheese shelf life, would you be in favour of their use?							0.00			0.00					0.51	
Yes	69.0	68.0	40.7$^{0.000}$	60.0	54.5	25.0		62.6$^{0.004}$	72.8$^{0.004}$		67.3	59.6	67.4	64.1		66.0
No	31.0	32.0	59.3$^{0.000}$	40.0	45.5	75.0		37.4$^{0.004}$	27.2$^{0.004}$		32.7	40.4	32.6	35.9		34.0
Q12 Would you be willing to pay more for the use of such technologies with packaged cheese products?							0.18			0.62					0.23	
Yes	25.7	27.0	13.0	12.0	27.3	12.5		25.1	23.5		23.7	18.0	28.3	23.4		24.6
No	74.3	73.0	87.0	88.0	72.7	87.5		74.9	76.5		76.3	82.0	71.7	76.6		75.4
Q13 Have you heard of any of the following terms?																
Smart Packaging							0.42			0.88					0.00	
Never heard of the term	58.6	50.6	61.1	64.0	72.7	62.5		57.9	56.3		62.3	53.9	57.2	47.7		57.4
Heard of the term but do not understand it	25.7	25.3	20.4	20.0	9.1	12.5		24.2	25.7		23.7	34.8	23.6	22.7		24.7
Heard of the term and understand the term.	15.8	24.2	18.5	16.0	18.2	25.0		17.9	17.9		14.0	11.2	19.2	29.7$^{0.000}$		17.9
Active Packaging							0.00			0.98					0.00	
Never heard of the term	79.7	68.5$^{0.001}$	81.5	92.0	81.8	62.5		77.8	77.2		83.5$^{0.001}$	79.8	75.4	66.4$^{0.001}$		77.6
Heard of the term but do not understand it	13.8	15.2	7.4	4.0	0.0	12.5		13.0	13.4		11.2	12.4	14.1	16.4		13.1
Heard of the term and understand the term.	6.5$^{0.000}$	16.3$^{0.000}$	11.1	4.0	18.2	25.0		9.2	9.3		5.3$^{0.002}$	7.9	10.5	17.2$^{0.001}$		9.2
Intelligent Packaging							0.03			0.92					0.00	
Never heard of the term	74.2	61.8$^{0.001}$	70.4	92.0	72.7	62.5		71.8	71.3		77.6$^{0.002}$	74.2	69.6	59.4$^{0.001}$		71.6
Heard of the term but do not understand it	16.2	21.9	13.0	4.0	9.1	12.5		16.8	16.4		13.7	16.9	18.5	20.3		16.7
Heard of the term and understand the term.	9.7	16.3	16.7	4.0	18.2	25.0		11.4	12.3		8.7	9.0	12.0	20.3$^{0.001}$		11.7
Nanotechnology							0.01			0.00					0.01	
Never heard of the term	36.1	24.2$^{0.001}$	44.4	56.0	45.5	25.0		42.1$^{0.000}$	19.4$^{0.000}$		36.4	34.8	38.4	21.9$^{0.001}$		34.6
Heard of the term but do not understand it	26.8	30.3	16.7	36.0	27.3	37.5		28.4	25.0		28.3	29.2	26.4	25.0		27.3
Heard of the term and understand the term.	37.2	45.5	38.9	8.0$^{0.002}$	27.3	37.5		29.5$^{0.000}$	55.6$^{0.000}$		35.2	36.0	35.1	53.1$^{0.000}$		38.1

[1]Education level abbreviations - Primary school or Secondary school (PS), Post leaving course, Further education and training course or an Apprenticeship (PFA), Third level certificate, Diploma or University degree (TDU), Masters degree, Postgraduate diploma, Doctoral degree or Higher Doctorate (MPDH).

P represents level of significance within a group. Superscript values indicate where this significance lies.

according to comments supplied by many of our respondents. Genetic modification was mentioned a number of times by respondents, which of course has nothing to do with the technologies pertinent to this survey, but highlights genuine areas of concern. It may also point to a general difficulty that technologists may have in attempting to propose new technological developments for application in food and beverage products when they become tainted by unrelated technologies possessing negative consumer connotations. Interestingly, Hagemann and Scholder (2009) suggested that consumers consider genetic modification to be the prototype for novel technologies. There was also suspicion raised over extending shelf-life and how this would be achieved. Chen *et al*. (2013) derived that the addition of preservatives and chemicals were often associated with very long shelf-lives. Many consumers were opposed to their inclusion, particularly if they were to be added directly to the cheese. However, consumers commented that if the additives or the technologies used were natural, then they would be more open to their use. Some respondents deemed the technologies proposed as being too risky and dangerous, and were particularly sceptical of their function and safety claim. Furthermore, the idea of interfering or tampering with food was considered unnatural. Cheese is viewed, for the most part, as a natural traditional product, and the more a product is seen as natural, like cheese, the less likely an engineered version of that product will be accepted (Tenbült *et al*., 2005). This evident aversion to the discussion of the possible use of smart packaging technologies in cheese products suggests this older segment of panellists is unlikely to accept any modifications that are technologically driven and thus, would avoid any cheese products containing such technologies. As alluded to previously, this could be a rejection of all technology, as opposed to a rejection of a specific technology (Frewer *et al*., 1997). Participants questioned the

real intention of the technology, implying that the majority of benefits were primarily for manufacturers and retailers and not for consumers. Additionally, the necessity of the technology was referenced, with several consumers proposing improving the primary packaging first, such as through the proper employment of resealability systems, more emphasis on storage instructions and reduction in pack and portion sizes. Despite the aforementioned issues, even those who had reservations about the necessity for smart packaging applications did admit that they understood the overall benefit of applying such technologies. If the technology was proven to be reliable and worthwhile, was demonstrated to be safe and was provided by a company participants trusted, then they would willingly be in favour of its use. Consumers were adamant, however, that the technology applied must not compromise or sacrifice cheese nutrition, taste or any other important sensory characteristics. Most respondents thought that the technologies proposed could only be an advantage, as any drawbacks considered were outweighed by; shelf-life extension, guarantee of a safer product and a decrease in undesirable spoilage, which would be especially useful if this problem could be circumvented after the cheese package was opened.

Overall, it can be stated that there was a general lack of knowledge regarding these technological terms, as shown in Table 4, with most consumers not having heard of the terms; active packaging (77.6%), intelligent packaging (71.6%) and smart packaging (57.4%). The exception to this was nanotechnology, with most participants understanding the concept. This may show that consumers are becoming more aware of such technologies, particularly when in recent years, consistent findings demonstrate that the public does not know much about nanotechnology (Besley, 2010). Significant differences were determined within age groups, however,

no specific trend was deciphered. There were no significant differences observed between males and females for responses to smart packaging, active packaging or intelligent packaging. For nanotechnology, males had a greater knowledge of the term, possibly due to a greater exposure to the 'Sci-Fi' genre or electronic gaming (derived from the response in Q16), and was significantly different to the responses obtained for 'never heard of the term' ($P \leq 0.000$) and 'heard of and understand the term' ($P \leq 0.000$). Within the educational profile employed in this survey, a general pattern emerged exhibiting that a higher level of education corresponded to an increased awareness and knowledge of these terms. Chen *et al*., (2013) also found that those possessing higher education levels were more likely to be aware, or familiar with new technology and were also more likely to try new things. This was reinforced by the significant differences experienced by panellist responses derived from PS and MPDH educational groupings. Those with a PS level of education had a lower awareness of the term active packaging ('heard of the term' – $P \leq 0.001$, 'heard of the term and understand the term' – $P \leq 0.002$) and for intelligent packaging ('heard of the term' – $P \leq 0.002$). Conversely, the highest level of education, MPDH, were the most aware and most knowledgeable of all technologies as demonstrated by the significant differences determined for the terms; smart packaging ('heard of the term and understand the term' – $P \leq 0.000$), active packaging ('heard of the term' – $P \leq 0.001$, 'heard of the term and understand the term' – $P \leq 0.001$), intelligent packaging ('heard of the term' – $P \leq 0.001$, 'heard of the term and understand the term' – $P \leq 0.001$) and nanotechnology ('heard of the term' – $P \leq 0.001$, 'heard of the term and understand the term' – $P \leq 0.000$).

Consumers were specifically asked if they had heard of these technological terms previously, how were the terms introduced to them, and was it in a positive or

negative context. Some individuals in the survey said they sought out knowledge due to an interest in the area, whereas, others were presented with the terms or inadvertently found them. Table 5 displays the forums of information discovery. Education and media were determined to be the main deliverers of information. Most consumers provided the context in which they had discovered the term, with many pointing out that they had not heard of these terms being used in relation to cheese. The themes mainly included preservation, medicine, science, technology, labelling and packaging, pharmaceutics, electronics, computing, engineering, cosmetics and future trends. Some contributed more specific examples of content and application such as; diagnostic imaging, disease prevention, particularly with coronal disease and cancer, dental materials, drug delivery, bread and meat preservation, modified atmosphere packaging, DNA repair, surveillance, sun cream, usage in cleaning up environmental disasters like oil spills, detergents, space travel, protein and fat manipulation, and electronics (robotics, fibre optics, semiconductors, nanoreactors, phones, computer chips, lasers, sensors and other smart systems and materials). Many respondents provided what they believed the function of the terms they recognized to be. The levels of understanding demonstrated elements of fact and mistruth, which shows that despite respondents stating that they were aware of these technologies, the level of awareness and actual knowledge may be misjudged by the participants. Koutsimanis *et al*. (2012) also noted a discrepancy between self-rated knowledge and actual knowledge of the consumer surveyed. Consumers were asked to describe the terms as positive or negative based on their experience or knowledge. Positive was mentioned 306 times with people who knew of the term or understood the term being open to the technology due to its ‘moderness’ and ‘efficiency’, and a number of individuals commented that the survey had peaked their interest and that

they had been encouraged to read more on the subject. Fewer participants expressed neutral (22) or negative views (39). The negativity expressed was focussed mainly on nanotechnology, with some commenting its portrayal in the media as often having negative connotations or undertones. Scepticism, potentially harmful and unknown effects on health, cost and an overall objection to integrating the technology with food, were the main negative feedback remarks, which is in contrast to the findings of Coles and Frewer (2013), who reported that at present, most people generally encapsulate a positive attitude towards nanotechnology.

Table 5 - Sources of information discovery

Education	*Secondary school subjects* - Home economics, Science
	PLCs, college, university and other institutues - agriculture, biochemistry, biology, biotechnology, chemistry, computer science, engineering, food business, food management, food science and technology, geography, grapic design, marketing, medicine, microbiology, nutrition, packaging, physics, technology, and others.
Media, Literature, Culture	*Magazines* - scientific, technological, food and medicinal magazines. E.g. Chemistry World, National Geographic, How It Works, Focus.
	Newspapers - Particularly the business section.
	Scientific journals
	Books - Both factual and fictional. *E.g.* Michael Crichton's Prey (fiction).
	Television - News, documentaries, science and technology based programs sci-fi and fantasy shows (Jimmy Neutron, Doctor Who, Star Trek).
	Advertisments - Specifically German, Australian and American participants mentioned campaigns promoting these technologies.
	Films - Spy and sci-fi genres (GI Joe: Rise of the Cobra).
	Video games - *E.g.* Metal Gear Solid and Red Faction.
	Internet - Food related websites, Youtube and the science and technology community, both websites and blogs (NewScientist and io9).
	Additional literature - Leaflets, bulletins and other science materials.
	Other sources - Radio, music (MC Doom), talks, showcases, workshops, schemes, open days, museums, debates, presentations, conferences.
Other	*Industry, individual companies and retailers.* *Through work.* *Friends or famillar.* *Printed on a product, label or packaging.* *Heard in passing or in conversation.*

2.3.5 Acceptance of smart packaging technologies

Information on all technologies was provided prior to evaluating acceptance, with only the function presented. No prior knowledge makes it hard for consumers to determine if the technology is acceptable or not. It is important to communicate positive aspects as acceptance relies on innovations that provide consumers with tangible and relevant benefits (Pilone *et al*., 2015), with applications related to food safety receiving the highest benefits scores (Siegrist *et al*., 2008). However, disseminating a benefit-only strategy may be problematic as the lack of disclosure may cause mistrust (Verbeke *et al*., 2008). A receptive attitude towards the incorporation of one or more technologies was determined (Table 6), with the overall most accepted technologies for consumers determined to be; incorporation of all three technologies (36.0%), combination of active and intelligent packaging (10.0%), and intelligent packaging only (9.2%). Younger participants were more accepting of all three technologies, with willingness to accept all three technologies decreasing with increasing age, and the preference for no technological interference higher for individuals over the age of 35. However neither of these trends were significant. There were no significant differences determined between genders and educational levels.

Intelligent packaging was most accepted because it contributed the least interference with cheese, compared to the other technologies proposed. Also, consumers felt that they had more control over the passive, yet functional, intelligent technologies. Respondents liked the idea of being able to use intelligent packaging systems to determine the condition of the food without needing to open the primary package. Pennanen *et al*. (2014) also confirmed that consumers are interested in the concept of intelligent packaging, consider the technology to be relevant and would be willing

Table 6 - Acceptance of technologies

	Age (%)						*P value*	Gender (%)		*P value*	Education (%)				*P value*	
	18 to 24	25 to 34	35 to 44	45 to 54	55 to 64	65 or older		Female	Male		PS	PFA	TDU	MPDH		Total (%)
Q17 Would you be willing to purchase a cheese product whose packaging has one or more of these technologies incorporated?							0.077			0.039					0.261	
Active Packaging only	6.5	6.2	3.7	4.0	9.1	0.0		6.8	4.9		5.6	7.9	7.2	3.9		6.1
Intelligent Packaging only	9.1	9.6	9.3	8.0	9.1	12.5		10.3	7.1		9.7	10.1	8.7	8.6		9.2
Nanotechnology only	2.4	3.9	7.4	4.0	0.0	12.5		2.4	4.9		3.1	3.4	1.8	6.2		3.2
Active and Intelligent Packaging	10.4	11.2	3.7	8.0	9.1	0.0		11.5	6.7		10.0	9.0	8.7	13.3		10.0
Active Packaging and Nanotechnology	4.1	3.4	0.0	4.0	18.2	0.0		4.2	3.0		5.6	0.0	2.9	3.9		3.8
Intelligent Packaging and Nanotechnology	5.6	6.2	11.1	4.0	0.0	0.0		5.3	7.1		4.7	6.7	5.4	9.4		5.9
Active Packaging, Intelligent Packaging and Nanotechnology	39.0	33.7	28.7	24.0	18.2	0.0		33.7	40.7		36.4	37.1	39.5	26.6		36.0
None of the above	22.9	25.8	37.0	44.0	36.4	75.0		25.8	25.7		24.9	25.8	25.7	28.1		25.8
Q18 Would you be willing to pay more for the use of such technologies with packaging cheese products?							0.466			0.802					0.210	
Yes	33.6	34.8	24.1	20.0	27.3	25.0		33.0	32.1		31.5	25.8	37.0	31.2		32.7
No	66.4	65.2	75.9	80.0	72.7	75.0		67.0	67.9		68.5	74.2	63.0	68.8		67.3

Education level abbreviations - Primary school or Secondary school (PS), Post leaving course, Further education and training course or an Apprenticeship (PFA), Third level certificate, Diploma or University degree (TDU), Masters degree, Postgraduate diploma, Doctoral degree or Higher Doctorate (MPDH).

P value represents level of significance within a group.

to adopt intelligent food packaging. Active packaging was deliberated to be appealing as it was seen to do the most to extend product shelf-life. In our previous question gauging consumer knowledge, active packaging was found to be the technology that respondents were least aware of. According to Brook Lyndhurst Ltd. (2009), people are generally most suspicious of the least familiar technologies. However, we observed an opposite trend, which was also demonstrated for nanotechnology whereby high familiarity correlated to a lower acceptance. For some, nanotechnology was preferred because it was the most well-known, expanded the performance range of existing pack materials, whilst deemed to use less resources. Although most respondents showed the highest level of awareness and knowledge of nanotechnology and there was a mostly positive response with regard to context; it displayed the least promising acceptance when it came to application. Perhaps the discussion of nanotechnology thus far, has been in abstract terms, and has received little exposure to actual applications (Siegrist *et al*., 2007). Cole and Frewer (2013) have also postulated that the use of nanotechnology in the food industry is closely associated to GM technology and the use of artificial additives, which may lead to similar rejection patterns. Innovations like active packaging or nanotechnology, could face rejection as they may reduce or be seen to conflict with the traditional image of cheese (Almli *et al*., 2011), therefore these areas in particular must proceed with caution when aiming for acceptance.

Compared to the majority of respondents being accepting of these technologies; just over a quarter (25.8%) of respondents rejected the use of any technology. The chief reasons for rejection were determined to be; consumers viewed packaging as an unimportant aspect of a product, viewed the extension of shelf-life as unnecessary, technologies were considered unsuitable for food and over the top for cheese,

implementation would come at a high cost to consumers, technologies would be inaccessible to small-scale cheese producers, benefits of using technologies accruing mainly to manufacturers and retailers, technologies were gimmicky and scary and packaging techniques were too new and not fully researched, therefore carrying unknown health and environmental risks, particularly for future generations.

For the most part, respondents commented that the technologies applied were less of an issue because the shelf-life extension was achieved using packaging methods as opposed to direct addition to the cheese itself, which is consistent with findings reported by Siegrist *et al*. (2008). Additionally, the type of cheese was considered a more important purchasing decision, and that they would have no concern with technology if it was surrounding a cheese they wanted to purchase anyway and as long as the characteristics of the cheese remained unaffected. Consumers thought these technologies would be particularly useful for a number of reasons – as cheese is an expensive product; protects cheese after opening; decreases food waste; convenient for many, but especially for single households, infrequent consumers, poorer households and for developing countries; suitable for soft cheese or grated cheese applications; allows for flexible storability and consumption can occur in a less concerned fashion; provides confidence for those worried about shelf-life dates and safety; cheese can be bought in bulk or larger portions for larger households or for frequent consumers, and advantageous for export within the dairy industry. Participants felt that the information provided made them feel more informed and intrigued. The information also triggered some consumers to recognize that they already have experience with this technology - use of thermochromic inks on Coors light beer bottles to indicate ideal drinking temperature. A portion of consumers were interested and excited about the prospect of the addition of innovative

technologies. They viewed technological advances as favoursome and just the next natural progression in preservation like salting, smoking or refrigeration.

2.3.6 Willingness to pay more for shelf-life extension

There have been conflicting reports on the importance of price on purchasing decision of products produced in conjunction with new technologies; some have said that the effect of price has limited importance (Rollin *et al*., 2011), while others have commented that price has a significant influence (Chen *et al*., 2013). At present, the implementation of smart technology accounts for a large portion of whole packaging cost, which is not realistically affordable by industry since packaging costs rarely exceed 10% of the total product cost. Nonetheless, it has been predicted that the packaging and technical process should decrease to a reasonable price level in the future (Dainelli *et al*., 2008). Our figures from Tables 4 and 6 (Q14 and Q18, respectively) and responses from participants showed that cost was a major contributor to purchasing decision. Consumers were in agreement with no significant differences determined between before (Q14) and after (Q18) receipt of information, that they would not pay more for the extension of shelf-life. However, it could be hypothesized that the 25.8% of respondents who rejected all smart packaging technologies outright in question 17, would be within the 'No' proportion of respondents (i.e. why would they pay more for technology that they did not want anyway). Therefore the level of 'No' responses from those that accepted shelf-life extension is probably much lower than reported. Despite the agreement between questions 14 and 18, the number of respondents willing to pay had increased, from 24.6% to 32.7%. Figure 1 compares willingness to pay for the use of smart

packaging technologies in cheese packs both before receiving information and after receiving information about these technologies. Significant differences were determined between the before and after responses ($P \leq 0.05$). This demonstrates that the provision of information on the technologies used to achieve shelf-life extension may positively influence purchasing decision. All respondent groupings within all categories increased their willingness to pay more at the point of purchase, with the exception of the 55 to 64 year olds. For both before and after receipt of information, 18 to 24 and 25 to 34 were the groups most likely to accept an increase in cost, which is in agreement with Colonna *et al*. (2011) who found as age increased, older individuals had a lower probability of purchasing a higher priced cheese. Females were more likely to pay an increased cost for an extension of shelf-life. This is most likely as they probably appreciate both the benefit of an extended shelf-life for storability purposes and lower cost due to the factors alluded to previously. No trend was deciphered with education, unlike Chen *et al*. (2013) who found Canadian consumers had an increased willingness to buy technology-driven packaging with increasing education levels. For the majority of respondents, the cost would need to remain the same, or even be reduced, as the price of cheese was already considered to be at a premium price point. Respondents understood that whilst there was the benefit of shelf-life for them through the employment of some of these technologies, they also pointed out the benefit accrued by the producers or retailers, was most likely a cost-benefit to these parties. Therefore, as previously reported by Frewer *et al*. (1997), they believed any cost benefit to the industry should be passed on to them, as an economic incentive for purchase since consumers have to trade off on the perceived risk for the implementation of such technologies (Ueland *et al*., 2012). Some consumers were willing to pay more for the additional time benefit. These

individuals may be inclined to pay more for a shelf-life benefit because it is their preferred attribute; whilst others would pay more as long as the shelf-life provided enough time to warrant an increased cost. They acknowledged while the price might be increased, that there is value in the long term, particularly for storability or bulk buying possibilities.

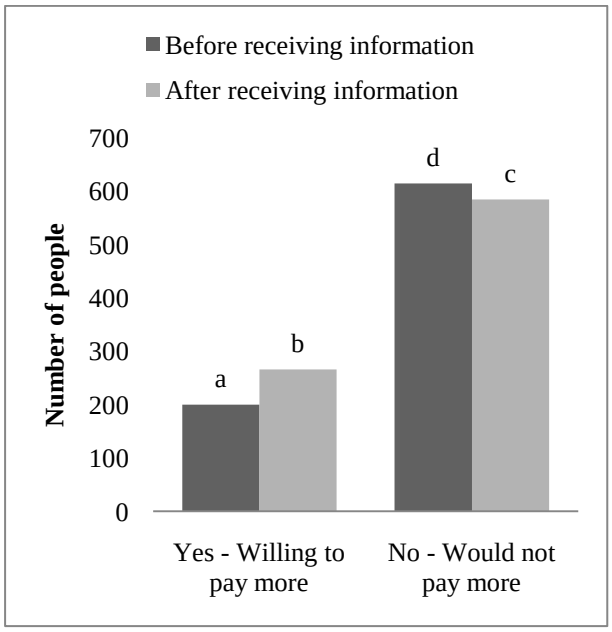


Figure 1 – Comparison of before and after receiving information responses regarding paying more for the implementation of smart technologies. A significant difference ($P \leq 0.05$) is indicated using lowercase lettering (a, b, c, d).

2.3.7 Future outlook

From this undertaking, attitudes towards the use of packaging technologies are mainly positive for the future of smart packaging technologies on fast-moving consumer products like cheese. However there is much to be done to garner widespread adoption by both consumers and industry. Due to the rapid development of these areas, further research and applications are needed to a gain a full understanding. All aspects of safety need to be established and the efficacy of the packaging technology needs be demonstrated as acceptance is highly dependent on the belief of the technology. Both industry and the public must also be reassured that these technologies will only reach the market after being subjected to assessment and granted regulatory approval by relevant authorities. It is important that this safety, research, function and regulatory approval be communicated to the public, as factual information has an alleviating effect on consumer's concern levels about technology (Cardello *et al*., 2003). The labelling or packaging surface may be the ideal vehicle to deliver information to the consumer, as it can; provide information whilst educating and raising awareness regarding the technology, demonstrate manufacturer transparency, prevent misuse or misunderstanding, and facilitate consumer choice, which is important should they wish to limit their exposure to a certain technology. However, labelling of products can negatively impact on the intention to purchase a product as observed with GMOs (Rollin *et al*., 2011). Whilst communication is important, and people can recognize the potential importance, unfamiliarity is an obstacle. Consumers need to be made aware of the fact that the concept of smart packaging is not a recent development and that they have been experiencing forms of this smart technology in packaging for many years, for example; colour changing inks, moisture pads, UV absorption materials in plastic packaging, susceptor

packaging for assisted product heating in microwave ovens etc. Applying the next level of smart packaging is just a natural progression in the evolution of packaging. A gradual introduction of smart packaging may be required as cheese packaging has rarely been subjected to change and any noticeable changes may face rejection. It is important that the new packaging is recognizable by the consumer, performs like the old packaging, and is compatible with consumer lifestyle (Ryynänen and Rusko, 2014). It is also important for consumers to experience the new technology in usage situations, as benefits perceived through tasting, handling and enjoyment, may change prejudiced perceptions of the products and serve as drivers for repurchase (Ueland *et al*., 2012).

2.4 CONCLUSION

This study found that cheese evokes a multitude of opinions; some individuals just consume cheese as a necessity, some individuals like cheese and consume it regularly and then there is a category of consumers, who have an intimate relationship with cheese and they do not just like cheese, but love it. The majority of the participants appreciated the benefits from the technology, but some questioned the appropriateness of its context, suggesting the need of an active function on current packaging as being redundant and unnecessary. In this instance they may be partially correct, and potentially the more evolved functions of smart packaging technologies should be targeted and used for specific applications. For example, more appropriate applications include; employment on soft or grated cheese, use with more premium and expensive cheese products, as a shelf-life extension mechanism that only begins once the cheese package is opened, or for use in various packaging systems where cheese products are being exported to distant international markets.

CHAPTER 3

Assessment of the influence of the industry distribution chain on the oxygen levels in commercial modified atmosphere packaged cheddar cheese using non-destructive oxygen sensor technology

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ABSTRACT

The establishment and control of oxygen levels in packs of oxygen-sensitive food products, like cheese, is imperative in order to maintain product quality over a determined shelf-life. Oxygen sensors quantify oxygen concentrations within packaging using a reversible optical measurement process and this non-destructive nature ensures the entire supply chain can be monitored and can assist in pin-pointing negative issues pertaining to product packaging. This study was carried out in a commercial cheddar cheese packaging plant and involved the insertion of 768 sensors into 384 flow-wrapped cheese packs (two sensors per pack) which were flushed with 100% carbon dioxide prior to sealing. The cheese blocks were randomly assigned and subjected to two different storage groups to assess the effects of package quality, packaging process efficiency, and handling and distribution on package containment. Results demonstrated that oxygen levels increased in both experimental groups examined over the 30-day assessment period. The group subjected to simulated industrial distribution route and handling procedures of commercial retailed cheese, exhibited the highest level of oxygen detected on any day examined, and also experienced the highest rate of package failure. The study concluded that fluctuating storage conditions, product movement associated with distribution activities, and the possible presence of cheese-derived contaminants such as calcium lactate crystals were chief contributors to package failure.

3.1 INTRODUCTION

Oxygen is directly or indirectly linked to the major incidences of spoilage associated with hard cheeses. Modified atmosphere packaging (MAP) utilises gas mixes deficient in oxygen to extend the shelf-life of hard cheeses by affording protection against oxidation and the proliferation of undesirable spoilage microorganisms. Consequently, hard and semi-hard cheeses are commonly surrounded by laminate combinations of polyamide and polyethylene (Schneider *et al*., 2010), and packed in 100% carbon dioxide or mixtures of carbon dioxide and nitrogen using horizontal form-fill-seal pouch pack equipment (Hotchkiss *et al*., 2006). The use of 100% carbon dioxide is favoured by many cheese packers as the employment of this MAP approach typically encourages inhibition of microbial growth, but more specifically, it produces a cheese pack with a physical appearance of a vacuum package. This occurs because carbon dioxide has a high solubility in high moisture/high fat foods at low storage temperatures, like cheese, and when applied in excess can result in a fully intentional package collapse (Parry, 1993) which is technically described as 'snug-back or snug-down'. This gas absorption equilibrium process (between the headspace and the product) is known to be relatively fast and is obtained within the first few days after packaging (Jakobsen and Bertelsen, 2002).

However, if the package, packaging process, storage or distribution conditions fail to contain hard cheese products properly, then the benefits imposed by modifying the atmosphere will be ineffective and 'snug-back or snug-down' will not occur, thereby negatively affecting the visual appearance of the final retail pack. Additionally, when such primary packaged cheese products have been manufactured for export, the primary packaging must contend with more challenging stresses from the point of collation, handling and distribution purposes, through extended distances and

environmental conditions presented as the primary packs move through the cold-chain distribution system employed in the market placement of such products. Therefore monitoring the level of oxygen within the package can give critical information on the status of cheese quality and shelf-life, and assist in the pin-pointing of negative containment issues as they arise from the point of product manufacture to the point of retail purchase.

Traditional methods used to determine the presence of oxygen within packaging headspaces are usually of a destructive nature. The tests are irreversible, products must be analysed in a batch-wise manner (continuous assessment cannot be achieved), and are unsuitable for identifying leaks. Non-destructive optical oxygen sensors quantify oxygen concentrations by measuring the luminescence quenching effect of oxygen (Smiddy *et al*., 2002). Sensors (solid supports containing phosphorescent dye) are monitored using a portable detector which emits a light source. This light source causes the sensor to become excited and simultaneously measures the changes in phase shift of the phosphorescence which is related to the quenching effect of excitation by oxygen (Papkovsky *et al*., 1995). The fundamental advantage of the employment of the sensors is that the measurement is a non-destructive reversible process, and therefore the entire supply chain can be monitored and control points can be identified.

Previously, O' Mahony *et al*. (2006) and Hempel *et al*. (2012) both evaluated oxygen levels using sensor technology in cheese packages (n=67 and n=40, respectively). However, both studies had small sample sizes and neither determined the influence of distribution on package function. This investigation concentrates on a larger sample size (384 cheese blocks) in an industrial setting and assesses the

influence of distribution on packaging containment by measuring oxygen levels in the cheese packages using non-destructive oxygen sensor technology.

3.2 MATERIALS AND METHODS

3.2.1 Oxygen sensor manufacture and calibration

Oxygen sensors based on a phosphorescent oxygen-sensitive dye (Platinum octaethyl porphyrin-ketone) spotted on microporous support (Hempel *et al*., 2013) were supplied by Luxcel Biosciences (Cork, Ireland). Each sensor (6 mm disc) was attached to the centre of an adhesive sticker (Avery Dennison, California, USA) (Figure 1 a). The sensor sticker was reapplied to the backing tape for mobility purposes and to maintain adhesion prior to package insertion. Sensors were calibrated using Optech® Platinum handheld reader (Mocon Inc., Minneapolis, USA) with two standard gas mixtures (0% oxygen and air), and the resulting calibration was stored in Optech® software and applied to the whole batch of sensors.

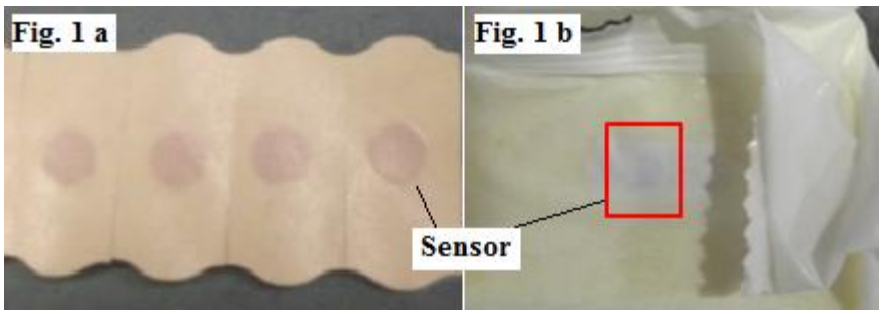


Figure 1 a and b – Sensor attached to an adhesive sticker (a) and sensor at the end of the cheese block within the cheese package (b)

3.2.2 Application of sensors

Sensors (n=768) were incorporated into cheese packs during the normal industrial packaging process at a local cheese packaging plant. The production line was slowed

down to allow two sensors to be placed directly on the cheese, at each end of the cheese block (Figure 1 b). Packages were made of orientated polyamide (15 µm)/polyethylene (50 µm), a laminate with low oxygen permeability properties. The cheese was packaged under standard packaging conditions - horizontal form, fill and seal, and flushed with 100% carbon dioxide prior to sealing. An online gas analyser (Dansensor, Ringsted, Denmark) read the initial oxygen levels to confirm that oxygen was not present in all packs. Packs that read an oxygen level greater than 0.5% were removed from the line and repackaged.

3.2.3 Experimental storage treatments

A total of 384 cheese blocks (~1 kg) were manufactured to contain the sensor technology in this study. Blocks were randomly designated to one of two experimental storage treatments, namely; A and B, with each cheese block assigned an identification number. Treatment groups A and B both contained 12 independent secondary corrugated paperboard boxes (160WTK/160SC/170T3 R Flute), each of which held 16 primary packaged cheese blocks stacked as per the usual structure employed for distribution formats (16 cheese blocks x 12 boxes = 192 cheese blocks per treatment). The two experimental storage groupings were as follows:

Group A –This group was stored under refrigerated conditions (4 °C) at the cheese packaging plant and subjected to minimal movement. Package fault could be due to a permeation issue or due to insufficient film thickness or it could be some aspect of the packaging process (alignment, gases applied, sealing) that is not executing its purpose. Therefore, storage group A examined the performance of the packaging process and package function.

Group B – Boxes were palletised and transported offsite via a commercial refrigerated (4 °C) truck and kept at an external storage facility overnight under refrigerated conditions. Palletised product was off-loaded and re-loaded using a forklift at the storage facility to further simulate commercial handling stresses. This group was then returned back to the original cheese packaging plant for assessment. The roundtrip distance experienced by cheese grouping B was approximately 100 km. Storage group B determined if packaging function becomes compromised through the conditions experienced during simulated transport and handling procedures which are experienced during the industrial distribution of retailed cheese.

3.2.4 Measurement of oxygen sensors

Oxygen measurements were recorded using an Optech® Platinum handheld reader (Mocon Inc.) which was connected to a laptop via a serial port. The Optech® detector acquires a measurement from an oxygen sensor within the package by placing the tip of the detector probe over the sensor (Figure 1 b). The readings were presented as % O_2 and stored in Optech® software.

Cheese packs were measured periodically for oxygen levels over a 30 day storage period. Both sensors in each pack were read and an average oxygen reading calculated. Table 1 details the measurement schedule for the trial. On day 0 (12 hr post packaging), boxes (1-11) in both storage groups were measured. Group A was monitored more frequently at the beginning of the trial, as any pack containment issues relating to packaging or processing were deemed most likely to occur earlier during the storage period. Box 12 in both groups was deemed a control box and was

served to determine if a lack of routine handling actually reduced the number of containment packaging faults experienced when compared with the more frequently handled packs in boxes 1 to 11. This box was measured only on day 30, which signified the end of the storage trial.

Table 1
Sensor measurement schedule

Day	Group A	Group B
0	(1-11)	(1-11)
2	(1-11)	x
9	(1-11)	(1-11)
16	(1-11)	(1-11)
23	(1-11)	(1-11)
30	(1-12)	(1-12)

Group A - Stored onsite and experienced minimal movement.
Group B - Subjected to simulated industrial distribution.
Boxes measured in brackets, with box 12 representing as a control.

3.2.5 Definition of cheese pack failure

A cheese package was deemed to have failed if pack containment was lost, as indicated by an oxygen content that was greater than 1%. Although complete elimination of oxygen is desired and processing procedures in the factory are implemented to remove all oxygen from packages, low levels of oxygen can become trapped within a package for various reasons during the packaging process and this oxygen is termed residual oxygen.

3.2.6 Pack integrity testing

Package integrity was performed after the conclusion of the 30-day trial period on all cheese packages which demonstrated failure (oxygen reading >1%). Integrity was assessed in two ways and these are described as follows:

- *Leak Detection System (PFM, Leeds, UK)*

The package was placed into the testing chamber and a vacuum pulled for 30 seconds at 0.6 bar. If bubbles were not present on testing, the cheese package was deemed to have 'passed'. However, if a constant stream of bubbles emerged from the package, then the pack was considered to have failed due to the presence of a leak, and the package was further inspected.

- *Submergence Test*

The leak detection test was augmented by carrying out the submergence test which involved the physical submergence of the packed cheese by an operator into a tank of water. This non-pressurised method allows the operator to manipulate the pack underwater. All pack seals were examined and small leaks inspected. Some light pressure was applied to certain pack areas when required in order to ensure that leaks were in fact present. If a leak was detected, the area of the leak was encircled using a permanent marker for further examination.

3.2.7 Headspace analysis

A commercial gas headspace analyser CHECK Checkmate® 990 (Dansensor) was used to establish progress within the package of the initial carbon dioxide applied

during the packaging process. It was also used confirm an oxygen level correlation with the non-destructive oxygen sensor measurements taken. This method was used only after day 30 of storage as it is a destructive evaluation and pack integrity becomes compromised post-assessment. The procedure involves applying a neoprene plastic pad to the package and piercing the package through the pad with the needle, ensuring the needle is exposed to the headspace without touching the cheese contained. The needle extracts a sample of gas from the headspace which is analysed for both oxygen and carbon dioxide levels.

3.2.8 Leak visualisation - Microscopy

A light microscope (Olympus BX61 – Mason Technology, Dublin, Ireland) was used to visualise and magnify any leaks detected in the packaging. The packaging materials surface was observed using reflected light.

3.3 RESULTS AND DISCUSSION

3.3.1 Measurement of oxygen sensors

Experimental storage groups A and B were monitored over a 30-day period and Figure 2 shows the mean oxygen content (%) of each group over this timeline. The oxygen level present in the cheese packs increased over time in both experimental treatment groups. In group A, the mean oxygen level for day 0 (0.02%) increased to 0.37% by day 30. Group B cheese packs had an initial oxygen level of 0.12% and this increased to a mean oxygen content of 1.05%. Our results are in agreement with Hempel *et al*. (2012), who observed an increase in oxygen within packs over a 148 hr trial period, and attributed this elevation due to poor packaging procedures. However, both treatment groups in this study were packaged identically, yet group B cheese packs had a higher oxygen content compared to Group A packs on any day examined throughout the storage period; thereby indicating clearly that distribution factors facilitates an increase in oxygen levels within cheese packs.

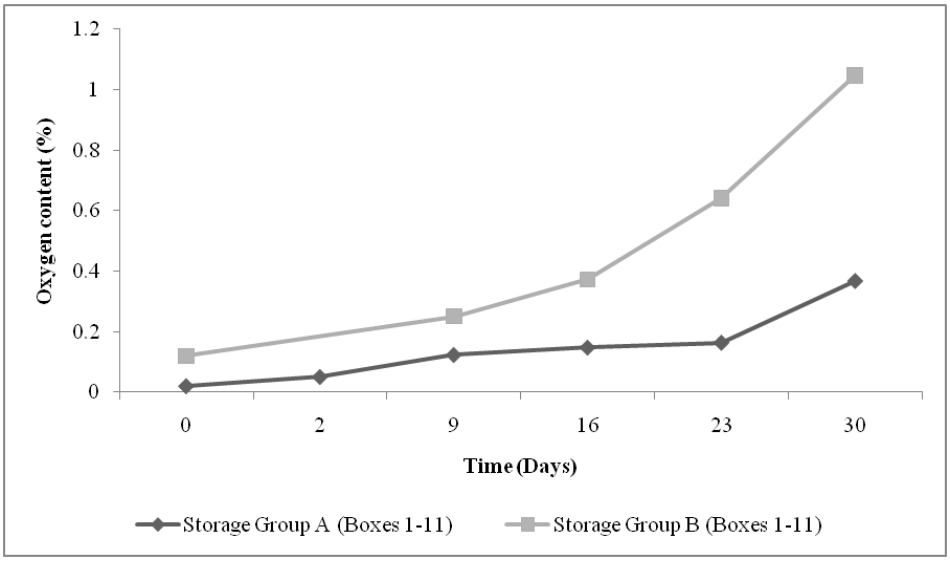


Figure 2 - Profile of the mean oxygen content of each storage treatment group (A - not distributed and B - distributed) assessed over 30 days.

3.3.2 Cheese Package Failure

The range-values for final oxygen levels within each treatment group are shown in Table 2, along with the overall % cheese pack failure for each experimental treatment. Group A cheese packs had a rate of failure of 3.13%, which indicated that the primary packaging and/or packaging process was not providing the full technical functions necessary to properly contain cheese packs. When compared to storage group A, group B cheese packs were found to have a pack failure rate of 7.29%; more than double the rate of pack failure observed for Group A cheese packs. Group B was the only storage group to leave the packaging facility and was therefore subjected to additional handling and transportation stresses, as well as being exposed to temperature fluctuations. Therefore, these distribution forces are the most probable factors in causing an increased level of containment failure. Rough transportation assists in accelerating the inception of oxygen by creating excessive movement which can cause friction between the cheese product and package, between each cheese package, between the packages and the corrugated paperboard box, and between the boxes and the pallet, plus any other tertiary packaging surround. In addition to interference caused by conveyance variations, temperature fluctuations can affect the package in two ways. Firstly, the permeability of packaging films is a function of temperature and permeability increases as temperature increases (Parry, 2013), which can result in containment failure. Secondly, at increased temperature, the solubility of carbon dioxide decreases and therefore, not only does snug-back fail to be achieved (affecting the visual appearance of the final retail pack), but the extent of antimicrobial activity is reduced as inefficient levels of dissolved carbon dioxide have been absorbed by the cheese,

thereby failing to provide the full antimicrobial capacity of the product (Papaioannou *et al*., 2007).

Table 2

Final oxygen ranges for both storage groups (no. of blocks) and rate of failure* (%) on day 30

Group	0-0.5%	0.5-1%	1-5%	5-10%	10-15%	15-20%	Total >1%	Failure (%)
A (192 blocks)	184	2	1	2	3	0	6	3.13
B (192 blocks)	173	5	1	2	10	1	14	7.29
Total (384 blocks)	357	7	2	4	13	1	20	5.21

* Failure is defined as levels that exceed 1% oxygen. The oxygen ranges underlined are deemed failures.

Group A - Stored onsite and experienced minimal movement. Group B - Subjected to simulated industrial distribution.

Additionally, it was hypothesised that the handling of the blocks, during the measurement procedure, could have contributed to the formation of leaks, thereby leading to more package failures and a false representation of results. Thus, one box in each of the two cheese groupings (Box 12) was used as a control and only measured once on day 30 of storage. Table 3 shows that failure occurred in this box for both storage groups A and B, despite never being opened and never being subjected to routine handling and measurement. This result demonstrates that handling the blocks during measurement minimally affected the rate of pack failure, and furthermore, oxygen sensors and the associated measurement process can be incorporated into an industrial setting without compromising package function.

Table 3

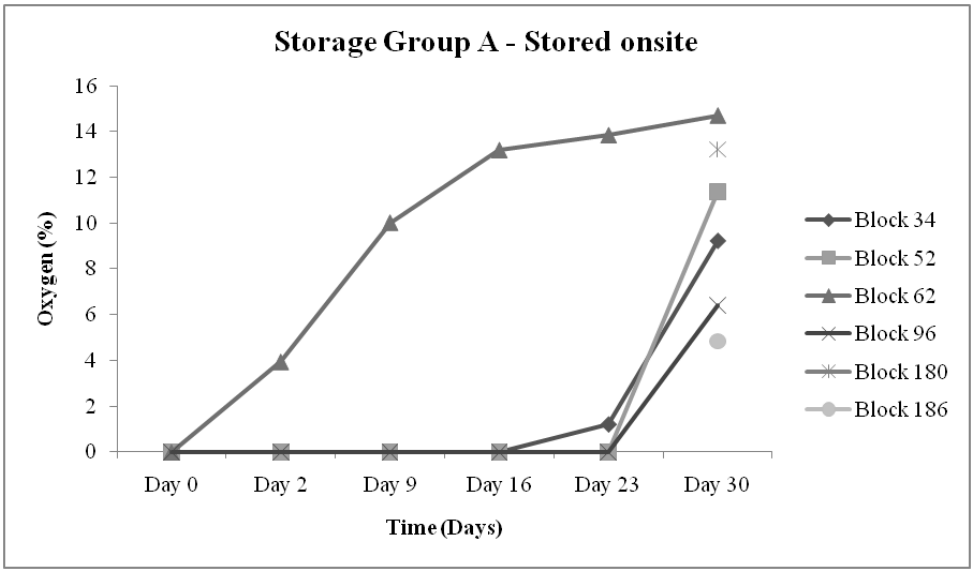
Box 12 (control) information

	Group A	Group B
No. of blocks in box	16	16
No. of failed blocks	2	1

Control box was never handled and only measured on day 30.

Group A - Stored onsite and experienced minimal movement. Group B - Subjected to simulated industrial distrubtion.

Figure 3 (a and b) displays the oxygen profile for the cheese blocks which exhibited failure (>1%) at any measurement point over the 30 days of refrigerated storage. A common feature of both of the oxygen profiles is that, in general, oxygen levels were under specified failure limits on the initial day of testing, with oxygen levels being low at the start of the trial and then experiencing a sudden spike in the oxygen content. This action may be explained by the presence of leaks as opposed to a permeation concern, as gradual increases in oxygen levels are more likely associated with poor permeability properties of the packaging material employed (Fitzgerald *et al*., 2001). Damage due to permeability involves changes in the gaseous atmosphere which occurs slowly; whereas leaks can cause rapid changes in the headspace and can also cause potential moisture and microbial ingression. O' Mahony *et al*. (2006) demonstrated that packs which presented increased oxygen contents, also demonstrated the earliest appearance of mould growth, which is usually not observed until much later. This shows that oxygen sensor measurement is key in predicting a reduced shelf-life and therefore, early detection is both informative and advantageous as failed blocks could be repackaged or removed from the supply chain, dependent on the stage of diagnosis.



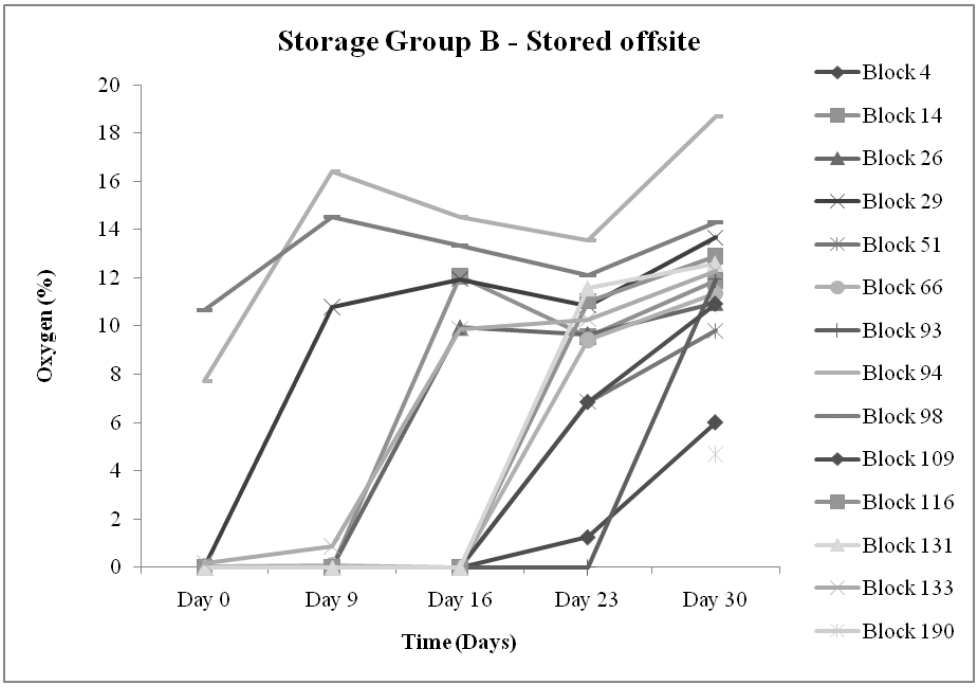


Figure 3 a and b - Shows the profiles of the progression of oxygen within the packs which diagnosed as failures (oxygen >1%) at any stage over the 30 day measurement period. Some failed blocks presented within box 12 (control) which was only measured on day 30. Each point represents the mean oxygen content of each failed pack on each measurement day.

3.3.3 Package Integrity Testing

The performance of the package and seal function was examined via package integrity tests. The cheese blocks assessed were those that were deemed to have failed (>1% oxygen levels in packs). These included 6 cheese blocks from group A (Blocks - 34, 52, 62, 96, 180, 186) and 14 cheese blocks from group B (Blocks - 4, 14, 26, 29, 51, 66, 93, 94, 98, 109, 116, 131, 133, 190). From visual assessment, the packs with the highest oxygen readings experienced a 'pillow' effect – a billowing or expansion of the package. This illustrates that oxygen had penetrated the inner atmosphere and it also indicated that the carbon dioxide applied during the packaging process potentially failed to be absorbed by the cheese.

Both integrity tests, which employed the use of water submergence, demonstrated the presence of bubbles emanating from a number of cheese packs. Often the existence of bubbles may indicate a false result and can be due to the occupancy of air entrapped within the seal-folds and at the seal-lips of the pack. Furthermore, if the bubbles present are arising from pack leaks, then detection of the leak can be extremely difficult depending on the pin-hole size. Medium- or large-sized pinholes can usually be observed by eye or detected easily via integrity testing. Smaller holes may be more harmful as they are much harder to identify and can evade detection (bubbles too small or infrequent from cheese packs during water submergence testing) and therefore, product deterioration can occur unknowingly. These small pores, microholes and cracks not only allow the transfer of gas and moisture from the external environment, but also permit microbial penetration into the package, with some bacteria penetrating holes as small as 0.4 μm in diameter (Robertson, 2013). The only visible fault detected was observed as a pinhole in group B (block 116). The pinhole position was on the main body on the back of the block; the hole

was marked (Figure 4 a) and later subjected to microscopy (Figure 4 b). This fault verifies that the integrity of the pack was compromised, and therefore any benefit bestowed by packaging in a modified atmosphere was lost. This concern was further explored by examining the headspace atmosphere.

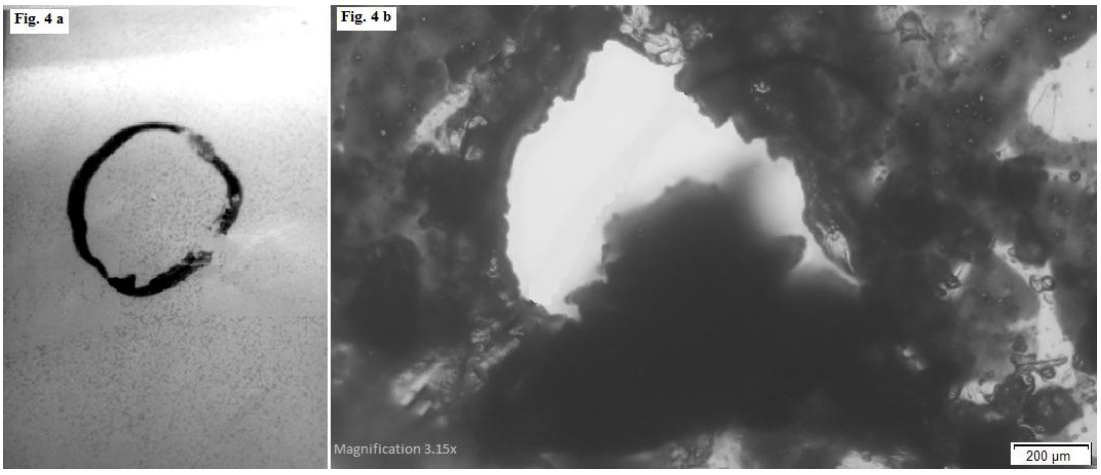


Figure 4 a and b - Marked location of the pinhole identified (a) and image of pinhole magnified using a light microscope (b).

3.3.4 Headspace Analysis

The failed packs were evaluated using headspace analysis post-integrity testing as this was a destructive method. This test was performed to confirm association between the two methods of oxygen measurement, and to ascertain what happens to the carbon dioxide within the package. Oxygen measurements were determined to be positively correlated with a correlation coefficient (R) of 0.7131, which indicates a strong relationship between oxygen sensor levels (non-destructive) and oxygen values obtained from the headspace analyser (destructive) (Figure 5). Since headspace analysis is of a destructive nature, this measurement was acquired after

the 30-day trial had concluded. Therefore, oxygen measurements taken by the gas analyser had increased levels, suggesting that the correlation may have been greater if the measurements of the two methods had been recorded simultaneously.

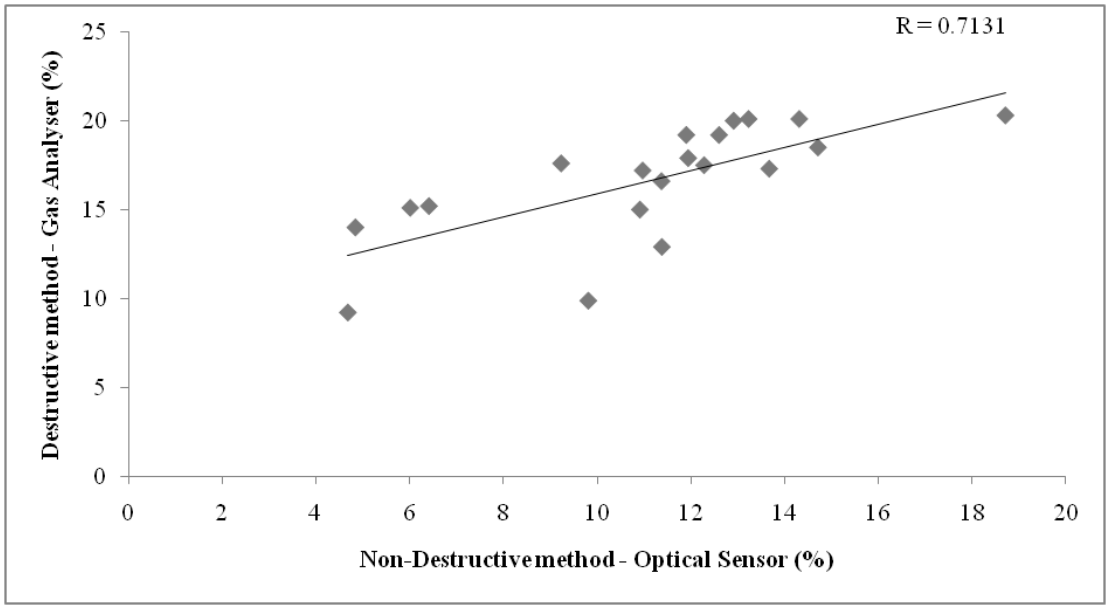


Figure 5 - Relationship between oxygen measurement methods (destructive and non-destructive) and their correlation coefficient value (R).

In general, the blocks that registered the lowest oxygen values correspond to the highest carbon dioxide levels (Figure 6). Blocks that were found to be failures exhibited a billowed appearance. The carbon dioxide levels within these packs were lower, which infers the carbon dioxide dissipates from the pack once the leak perpetuates and oxygen continually ingresses. Therefore, the benefit of microbial protection provided by carbon dioxide was lost and the progression of oxygen into the pack would have lead to a shortening of shelf-life and a deterioration in product quality.

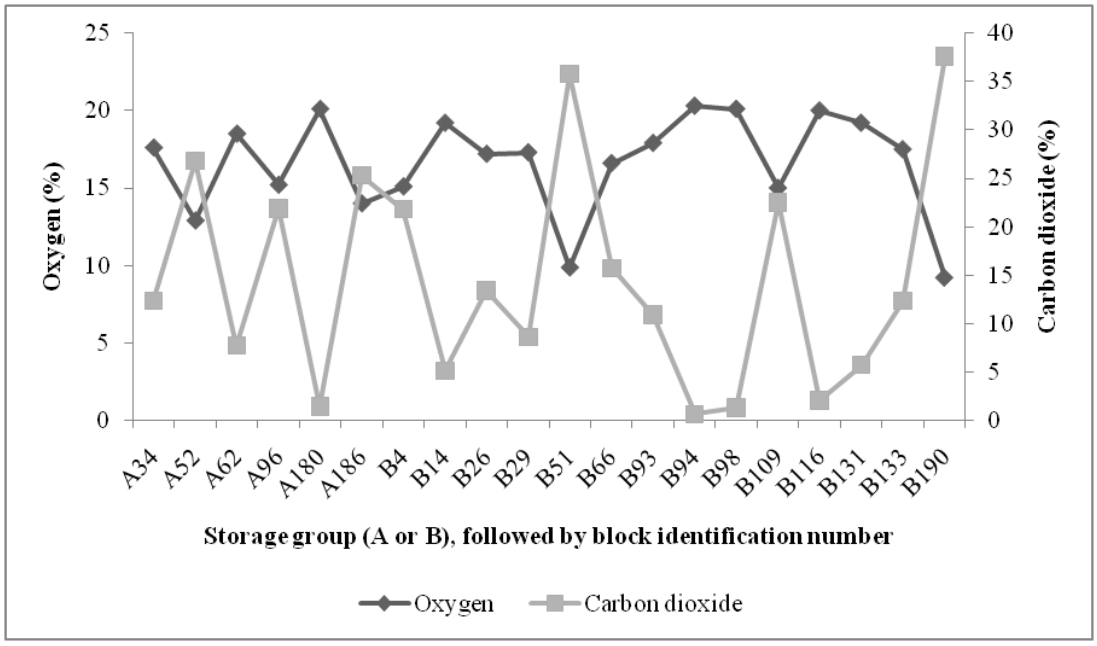


Figure 6 - Comparison of carbon dioxide and oxygen readings of failed cheese blocks from storage groups A and B.

3.3.5 Microscopy of Packaging Faults

The marked location where the pinhole was discovered through pack integrity testing was visualised using a light and fluorescence microscope (Figure 4 b). From physical examination of the pack, it was observed that pinhole formation emanated from the inside of the package, indicating that an entity within the pack was most likely to be the causative agent. Features within the pack which may be responsible include; sharp edges presented by the cheese itself from cutting the cheese into blocks, coarseness on the block surface or the presence of lactate crystals on the exterior of the cheese. The position of our pinhole on the cheese surface suggests that crystals were the most probable cause due to their size, morphology and potential to create small holes. Crystals in cheese can be both desirable and undesirable. Some cheese manufacturers produce cheeses with a higher lactate crystal content owing to the

distinctive sensory profile created by their presence. Calcium lactate crystal formation is influenced by cheese characteristics (manufacturing, shape, maturity, composition, bacteria present), packaging (format, integrity, barrier), storage and distribution conditions (temperature, light exposure and excessive handling), and in particular, it has been noted that gas flushing with carbon dioxide alone can cause faster and greater generation of crystals (Dybing *et al*., 1988). Therefore, a modified atmosphere of 100% carbon dioxide and an increase in temperature or movement, can all catalyse crystal production. Storage group B was specifically subjected to simulated distribution, and the conditions it was exposed to, coupled with their associated promotion of crystal formation, may have contributed to its high rate of failure. Such high levels of failure are unacceptable from a commercial standpoint and this could mean significant losses for the cheese industry unless resolved. Approaches could be implemented to reduce crystal development in order to minimise these losses from occurring, however, due to certain manufacturers targeting the increased existence of these crystals, an alternative approach is required. Therefore, there is a need to carefully monitor and control the conditions that occur during packaging, handling, storage, and distribution of hard cheeses in order to avoid excessive product losses owing to pack containment failures. Engagement of both active and intelligent systems, specifically the employment of oxygen sensors, can aid in the regulation of the entire supply chain.

3.4 CONCLUSION

As demonstrated in this study, sensors can be utilised successfully at an industrial scale to monitor the extent of abuse, from packaging onwards and allow changes to be made to the supply chain following the gathering of information. The mean oxygen content in both experimental storage groupings increased over time. The highest rate of failure occurred in group B with the source of failure determined to be due to the presence of leaks. The predominant reason for the formation of leaks was found to be due to the distribution conditions experienced and the possible existence of crystals on the cheese surface. Future work should focus on; implementing greater control throughout the supply chain to avoid the occurrence of leaks, the incorporation of these non-destructive sensor systems which are key in recognising when leakage does occur and the employment of smart technologies like antimicrobial packaging which can provide additional protection should a leak manifest.

CHAPTER 4

Assessment of the antimicrobial activity of potentially active substances (nanoparticled and non-nanoparticled) against various spoilage and cheese-derived microorganisms

Karen A. M. O' Callaghan and Joseph P. Kerry

This Chapter is in the form of an accepted manuscript and published in the *International Journal of Dairy Technology* (2014).

ABSTRACT

This study evaluated the antimicrobial potential of various naturally occurring substances, for use as active agents in cheese packaging. Sorbic acid and benzoic acid were examined as standard-sized and nanoparticled solutions. Rosemary, curcumin and ascorbic acid were employed as nanoparticled solutions only, while non-nanoparticled chitosan, of two molecular weights, was also selected for evaluation. All agents were assessed against a selection of Gram-positive and Gram-negative microorganisms, as well as cultures derived from cheese. Chitosan proved to be the most effective, with low molecular weight chitosan performing best. The most antimicrobial nanoparticle was found to be rosemary. Comparison of normal- and nanoparticle-sized organic acid solutions showed little difference in terms of antimicrobial properties.

4.1 INTRODUCTION

Cheese is a dairy product known for its many varieties, range of flavours and textures, nutritional value and its diversity as an ingredient. Cheese consumption can be an important measure of dietary quality in a country (Beijing Orient Agribusiness Consultant Ltd., 2011), and traditionally it is seen as an expensive commodity. However, consumption is growing in areas like Africa, Asia, Central and South America, and Eastern Europe, traditionally places where dairy product consumption has been low. Consumption in Russia, Brazil and Argentina is rising on average by 5–7% annually and in Mexico and South Korea by about 3% (Hollister, 2011). A boost in economic growth, rapid urbanisation, increased use of refrigeration and the globalisation of the western diet are contributing to this increase in dairy product consumption (Donnellan *et al*., 2011). Access to new markets requires transport over long distances and appropriate distribution and storage channels; central to achieving this is the selection and application of appropriate packaging materials and systems.

Conventional packaging materials used for cheese products include glass, metal, wax, paper, wood, plastics and laminates, using formats such as modified atmosphere packaging (MAP), vacuum packaging, sealed trays, cans, bags and wraps, squeezy bottles, tubes, casings, jars, boxes and tubbed products. Traditional materials and formats are employed to control moisture and provide resistance to gas exchange, thereby discouraging microbial growth, oxidation, discolouration and an overall negative sensorial impact. However, the environments under which cheese is globally exported include increased or fluctuating temperatures and humidities and excessive movement and handling. All of these can increase microbial activity, impact on numerous quality attributes and shorten shelf-life. To increase shelf-life or at a minimum, maintain a standard quality throughout exportation periods, more

suitable packaging materials and systems must be developed to cope with the challenges presented to cheese products. The development of active packaging systems for use with cheese products may be one such approach. This would serve as second-level packaging technologies, augmenting first level conventional packaging in a manner which would make it more functional by being more preservative in nature (Kerry, 2013).

Antimicrobial packaging materials extend the lag period and reduce the growth rate of microorganisms, helping to maintain product quality and safety and enforce an extended shelf-life (Han, 2000). Organic acids, spices and herbs, and polysaccharides are commonly used as preservatives in antimicrobial packaging. Organic acids occur widely in nature and have been frequently used in food preservation because of their wide spectrum of activity against a large range of microorganisms (Baird-Parker, 1980; Sofos and Busta, 1983; Hismiogullari *et al.*, 2008). The salt derivatives of organic acids are often used instead as solubility of organic acids can be an issue. Spices, herbs and other natural components have been used as preservatives since antiquity; however, in recent years, they have received a resurgent boost as potentially active agents in smart packaging applications. This increased focus is due in part to their naturalness, abundance in nature, non-toxicity, and chiefly for their inherent antimicrobial and antioxidant properties. The antioxidant activity of curcumin and rosemary has been well established (Sreejayan and Rao, 1996; Erkan *et al*., 2008), and other studies have shown valid antimicrobial action against various microorganisms (Shelef *et al*., 1980; Baratta *et al*., 1998; Wang *et al*., 2009). Chitosan is a polysaccharide that is naturally abundant, nontoxic and biodegradable (Li *et al*., 1997). It has a broad spectrum of antimicrobial, antifungal and antioxidant activity (Wang, 1992; Roller and Covill, 1999;

Xie *et al*., 2001). The antimicrobial activity of chitosan depends on its molecular weight and other factors like the degree of deacetylation and extent of chemical degradation (Lee, 2005).

Nanotechnologies involve the use of manipulative techniques on matter at a very small scale, generally between 1 and 100 nanometres (Cushen *et al*., 2012). When particle size is reduced below this threshold, the resulting material exhibits physical and chemical properties that are significantly different from the properties of macroscale materials composed of the same substance, which is particularly significant in the application of antimicrobial food packaging (Duncan, 2011). The novel properties and phenomena displayed by these nanoparticles include increased solubility and improved reactivity, which contribute to an overall enhanced efficacy. Particles at this scale can also infer mechanical, thermal and physio-chemical properties and improve the barrier properties (gas, moisture, UV light) of the overall packaging material (Sorrentino *et al*., 2007).

The aim of this study was to assess the antimicrobial activities of a range of different compounds (nano-sized and non-nanoparticled in nature) with the potential to inhibit the growth of a selection of bacteria including cheese (cottage cheese and Emmental) -derived cultures. The study compared the activity of standard-sized organic acids to commercial nano-sized equivalents. Non-nanoparticled chitosan of two molecular weights, low molecular weight chitosan (50 000–190 000 Da) and medium molecular weight chitosan (190 000–310 000 Da) were examined to determine if molecular weight influences the level of activity. Also assessed was the antimicrobial activity of other additional nanoparticled solubilisates – curcumin, rosemary extract and ascorbic acid.

4.2 MATERIALS AND METHODS

4.2.1 Materials and microbiological media

Standard-sized sorbic acid and benzoic acid, and both chitosans, low molecular weight (50–190 kDa) and medium molecular weight (190–310 kDa) were sourced from Sigma-Aldrich Co., St. Louis, MO, USA. All nanoparticled solubilisates (4% sorbic acid, 12% benzoic acid, 6% curcumin, 6% carnosic acid (rosemary extract), 10% ascorbic acid) were obtained from Aquanova AG, Darmstadt, Germany. A solubilisate is a colloidal liquid carrier solution containing an encapsulated active substance in an ultrafine distributed micelle structure (~30nm). The active component within the product micelle remains chemically unchanged. They are of food grade, both water and fat soluble, and are thermally, mechanically and pH stable. Acetic acid (Fisher Scientific UK Ltd., Leicestershire, UK) was used to improve the solubility of chitosan. All media, Tryptone Soya Agar (TSA) and Mueller-Hinton Broth (MHB) were acquired from Oxoid Ltd., Basingstoke, Hampshire, UK. Antimicrobial activity was measured using 96-well tissue culture microplates (Sarstedt, Inc., Newton, NC, USA).

4.2.2 Microbial strains and growth conditions

The microorganisms used for the antimicrobial sensitivity testing included the Gram-negative species, *Escherichia coli* (NCIMB 11943) and *Pseudomonas fluorescens* (NCIMB 9046), and the Gram-positive species, *Staphylococcus aureus* (NCIMB 13062) and *Bacillus cereus* (NCIMB 9373). These strains were cultivated on TSA slants and then maintained and stored at 4 °C. Microbial cultures were regenerated

twice from TSA slants into sterilised MHB and incubated for 18 hr at 30 °C or 37 °C, depending on the bacterial species. Prior to estimation of the minimum inhibition concentration (MIC), the concentration of these inoculums was determined to be 10^8-10^9 colony forming units (CFU) per ml. General mixed cheese cultures were derived from both Emmental and cottage cheese samples. Emmental cultures were prepared by homogenising 10 g of cheese with 90 ml of MHB in a Colworth Stomacher 400 (Seward Ltd., Worthing, UK). Emmental homogenate (1 ml) was transferred into 10 ml MHB, and then, the sample was incubated for 18 hr at 37 °C (10^6 CFU/ml). Cottage cheese culture was prepared by swabbing the surface of the cheese and transferring the swab into 10 ml MHB and incubated for 18 hr at 37 °C (10^7 CFU/ml), prior to testing. Cottage cheese could not be prepared in the same manner as Emmental as the homogenate was too cloudy to employ in MIC testing, which is dependent on visual results.

4.2.3 Antimicrobial preparation

The antimicrobials chosen for screening are listed on Table 1, as well as their preparation conditions. Both standard-sized organic acid solutions were prepared at 0.25% (w/v) due to their solubility. The solubility of sorbic acid and benzoic acid in water is 2.5 g/l at 30 °C and 2.9 g/l at 20 °C, respectively (Budavari, 1996). The nanoparticled sorbic acid and benzoic acid solubilisates were prepared at 0.25% (w/v), which allows for direct comparison with the standard sorbic and benzoic acid solutions. The rosemary and ascorbic acid nanoparticles were prepared at 1% (w/v). The nanoparticled curcumin solubilisate was set at 0.2% (w/v) as recommended by the suppliers. Chitosan solutions were prepared in a 1% (v/v) acetic acid solution,

with both the low and medium molecular weight chitosan prepared at a level of 0.25% (w/v). A control of 1% (v/v) acetic acid in sterile distilled water without chitosan was used to determine if the acetic acid contributed to any antimicrobial effect demonstrated by the chitosan.

Table 1

Antimicrobial agents and their magnitudes.

Agents	Abbreviation	Nanoparticled	Non-Nanoparticled	Concentration (%)	Dissolution Temp. (°C)	Solvent
Sorbic Acid Solution	SASN		✓	0.25	30	Water
Sorbic Acid Solubilisate	SASB	✓		0.25	40	Water
Benzoic Acid Solution	BASN		✓	0.25	20	Water
Benzoic Acid Solubilisate	BASB	✓		0.25	40	Water
Ascorbic Acid	As.A	✓		1	40	Water
Curcumin	CUR	✓		0.2	40	Water
Rosemary	ROSE	✓		1	40	Water
Low Molecular Weight Chitosan	LMWC		✓	0.25	20	Acetic acid & water
Medium Molecular Weight Chitosan	MMWC		✓	0.25	20	Acetic acid & water
Acetic Acid	AA		✓	1	20	Water

4.2.4 Antimicrobial susceptibility assessment

The antimicrobial action of the prepared agents was evaluated by determining the MIC against the various microbial strains using the micro-dilution method and 96-well tissue culture microplates as outlined by Figure 1. Volumes of 100 µl of the growth medium, sterile MHB, were pipetted into rows A to F, 1–12, with an additional 200 µl of MHB into H 12. Aliquots of 150 µl of the antimicrobial solution were dispensed to row G. Row H, 1–11, contained 200 µl of the test culture. A volume of 50 µl of the antimicrobial in row G was taken, transferred and mixed into row F. Subsequently, 50 µl of the resultant mixture from row F was extracted and then transferred and mixed into row E. This same procedure was repeated to row B, thus creating a threefold serial dilution. A volume of 50 µl was taken from row B and this was discarded. Following dilution, each well in rows A to G was inoculated with 15 µl of the microbial culture from row H. Column 12 contained no culture and represented a no-growth control. Row A contained no antimicrobial and was

therefore used as a positive growth control. The microplate was incubated for 18 hr at 30 °C for *P. fluorescens* and *B. cereus* and at 37 °C for *E. coli*, *S. aureus* and both cheese-derived cultures. Following incubation, microbial growth was identified visually as turbidity in the plate wells, and MIC was defined as the lowest concentration of antimicrobial agent showing a complete growth inhibition of the test cultures and expressed as percentage (w/v) inhibition values.

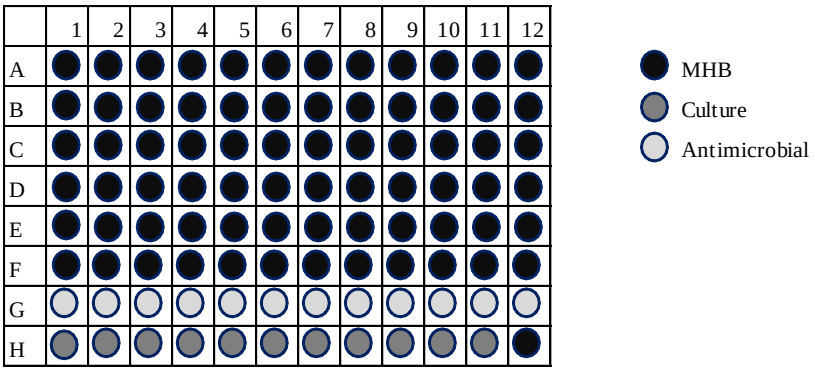


Figure 1 - 96-well Tissue Culture Microplate.

4.2.5 Statistical analysis

The experiment was performed twice: the first time in triplicate and the second experiment in duplicate. The total number of data points for each antimicrobial agent being five. The experimental data were analysed on SPSS Statistics 20 (IBM, Armonk, NY, USA). Data were presented as mean values ± standard deviation. Significant differences within a treatment was determining by using ANOVA. Paired *t*-tests were used to determine statistical significance between treatment means. Statistical significance was defined as $P \leq 0.05$–0.01 (significant), $P \leq 0.01$–0.001 (highly significant) and $P \leq 0.001$ (extremely significant). Means with the letters 'ns' are non-significant, $P > 0.05$.

4.3 RESULTS AND DISCUSSION

4.3.1 Antimicrobial potency of antimicrobial agents

The mean MIC (%, w/v) and standard deviations of the test samples treated with various antimicrobial agents are shown in Table 2. All antimicrobial agents, with the exception of the curcumin and ascorbic acid solubilisates, exhibited antimicrobial activity on one or a number of test cultures. Both cheese-derived microbial samples showed numerical reduction. The cottage cheese-derived microflora was inhibited by all working antimicrobials – standard solutions of sorbic acid and benzoic acid, nanoparticled sorbic acid, benzoic acid and rosemary, low molecular weight chitosan, medium molecular weight chitosan and acetic acid. Emmental-derived microflora was reduced through the use of a standard solution of benzoic acid, rosemary, both chitosan forms and acetic acid. Gram-positive microorganisms, *S. aureus* and *B. cereus*, also experienced inhibition, with four and five active agents causing an antimicrobial effect, respectively. The agents demonstrating antimicrobial activity against both Gram-positive strains were rosemary, both chitosan forms and acetic acid. Standard solutions of benzoic acid had an additional effect on *B. cereus*. Both Gram-negative species, *E. coli* and *P. fluorescens*, were least affected by all of the agents used, with only the chitosans and acetic acid causing a decrease in bacterial levels.

4.3.2. Comparison of non-nanoparticled solutions and nanoparticled solubilisates – Sorbic Acid and Benzoic Acid

When non-nanoparticled sorbic acid and benzoic acid solutions were compared to their equivalent nanoparticled sorbic acid and benzoic acid solubilisates, only a marginal difference in action was observed, with a slightly more favourable result found for standard-sized solutions. No differences in action were determined between non-nanoparticled sorbic acid and nanoparticled sorbic acid. Similar results were found for non-nanoparticled benzoic acid and nanoparticled benzoic acid, with two exceptions, Emmental-derived cultures and *B. cereus*. Non-nanoparticled benzoic acid had lower MIC's for Emmental (0.116%) and *B. cereus* (0.206%) compared with those recorded for nanoparticled benzoic acid (0.250%) (Table 2). From statistical analysis, only the difference in action against Emmental-derived microflora was significant ($P \leq 0.05$) (Table 3). Additionally, of the two non-nanoparticled solutions, antimicrobial activity was similar, except against the Emmental culture whereby benzoic acid performed significantly better than sorbic acid ($P \leq 0.05$) (Table 3).

The antimicrobial activity of organic acids is thought to be primarily due to a disruption of membrane function and key enzymes (Dillon and Cook, 1994). Organic acids inhibit microorganisms by interfering with the permeability of the microbial cell membrane, causing uncoupling of both substrate transport and oxidative phosphorylation from the electron transport system (Freese *et al.*, 1973). The uncoupling of these systems results in the acidification of the microbial cell contents. It was assumed that the solubilisates, due to their encapsulated character and nano-scale, would achieve a greater penetration and potency than the conventional, non-nanoparticled solutions, as shown in a previous study (Cruz-Romero *et al.*, 2013);

Table 2 Mean minimum inhibition concentration (MIC) (%, w/v), standard deviation and significance of test antimicrobials against cultures.

Antimicrobials	Emmental Cheese		Cottage Cheese		*Escherichia coli*		*Pseudomonas fluorescens*		*Staphylococcus aureus*		*Bacillus cereus*		Overall MIC
Sorbic Acid Solution 0.25%	0.250 ± 0.000	ns	0.116 ± 0.075	*	0.250 ± 0.000	ns	0.250 ± 0.000	ns	0.250 ± 0.000	ns	0.250 ± 0.000	ns	0.228
Sorbic Acid Solubilisate 0.25%	0.250 ± 0.000	ns	0.116 ± 0.075	*	0.250 ± 0.000	ns	0.250 ± 0.000	ns	0.250 ± 0.000	ns	0.250 ± 0.000	ns	0.228
Benzoic Acid Solution 0.25%	0.116 ± 0.075	*	0.116 ± 0.075	*	0.250 ± 0.000	ns	0.250 ± 0.000	ns	0.250 ± 0.000	ns	0.206 ± 0.099	**	0.198
Benzoic Acid Solubilisate 0.25%	0.250 ± 0.000	ns	0.116 ± 0.075	*	0.250 ± 0.000	ns	0.250 ± 0.000	ns	0.250 ± 0.000	ns	0.250 ± 0.000	ns	0.228
Curcumin Solubilisate 0.2%	0.200 ± 0.000	ns	0.200 ± 0.000	ns	0.200 ± 0.000	ns	0.200 ± 0.000	ns	0.200 ± 0.000	ns	0.200 ± 0.000	ns	0.200
Rosemary Solubilisate 1%	0.111 ± 0.000	***	0.037 ± 0.000	***	1.000 ± 0.000	ns	1.000 ± 0.000	ns	0.111 ± 0.000	***	0.037 ± 0.000	***	0.383
Ascorbic Acid Solubilisate 1%	1.000 ± 0.000	ns	1.000 ± 0.000	ns	1.000 ± 0.000	ns	1.000 ± 0.000	ns	1.000 ± 0.000	ns	1.000 ± 0.000	ns	1.000
Low Molecular Weight Chitosan 0.25%	0.028 ± 0.000	***	0.028 ± 0.000	***	0.050 ± 0.300	ns	0.028 ± 0.000	***	0.083 ± 0.000	***	0.050 ± 0.030	*	0.045
Medium Molecular Weight Chitosan 0.25%	0.072 ± 0.025	**	0.028 ± 0.000	***	0.083 ± 0.000	***	0.039 ± 0.025	*	0.116 ± 0.075	*	0.061 ± 0.030	*	0.067
Acetic Acid 1%	0.111 ± 0.000	***	0.111 ± 0.000	***	0.111 ± 0.000	***	0.096 ± 0.033	**	0.466 ± 0.298	*	0.096 ± 0.033	**	0.165

Values correspond to mean data for five test samples each, ± corresponds to the standard deviation. Within each row, means with an asterisk indicate significance.

The level of significance is denoted by the number of asterisk's present, * = $P \leq 0.05$ - 0.01 (significant), ** = $P \leq 0.01$ - 0.001 (highly significant), *** = $P \leq 0.001$ (extremely significant). Means with the letters 'ns' are non-significant, $P > 0.05$.

Table 3 Paired T-Test - Comparsion of solutions and solubilisates.

Pairs		Difference	Significance
SASN	SASB	No Difference	
BASN	BASB	Emmental	*
		B. Cereus	ns
SASB	BASB	No Difference	
SASN	BASN	Emmental	*
		B. Cereus	ns

The table describes the statistical differences computed.

The level of significance is denoted by these asterisks;

* = P ≤ 0.05 - 0.01 (significant), ** = P ≤ 0.01 - 0.001

(highly significant), *** = P ≤ 0.001 (extremely significant).

Means with the letters 'ns' are non-significant, P > 0.05.

however, the results presented in this study contradict this hypothesis. Low levels (0.25%) of the nanoparticled solubilisates were examined because of the low solubility of standard-sized solutions in water. An increase in solubilisate level may help extend the activity range and explain the difference in action reported by Cruz-Romero *et al*. (2013) with that detailed here. Similarly, Wendorf *et al*. (2008) found no differences between microparticle and nanoparticle formulations when used in vaccine delivery systems.

4.3.3 Examination of the nanoparticled solubilisates – Curcumin, Rosemary and Ascorbic Acid

It can be postulated from Table 2 that both curcumin and ascorbic acid solubilisates demonstrated no antimicrobial effect. Curcumin has been used as a natural colourant in the past for food and non-food applications; however, to achieve a colourless solution (as specified by the commercial supplier of the nanoparticled curcumin solubilisate), a 1:500 dilution with water is required. The use of a clear curcumin solution in this study was imperative as microbial inhibition through MIC was measured visually. Consequently, 0.2% of a curcumin solution was used, and this level may not have been high enough to incur an antimicrobial effect. The high dilution may have created a neutral environment, conducive to bacterial growth. If visual assessment was not the method of determining activity, an increased level may have provided alternative results to those achieved in this present study. As previously described, ascorbic acid is primarily employed in food systems as an antioxidant, with its use as an antimicrobial being less established. Tajkarimi and Ibrahim (2011) found that the application of non-nanoparticled ascorbic acid alone

had very little effect in decreasing *E. coli* in laboratory media and in carrot juice. This is in agreement with our results in which nanoparticled ascorbic acid propagated no antimicrobial effect. As with ascorbic acid, rosemary solubilisates have also been used as antioxidants in food systems. However, unlike ascorbic acid, rosemary was found to exhibit antimicrobial capabilities. In this study, rosemary exerted strong antimicrobial activity against mixed microbial cultures derived from Emmental and cottage cheese with mean MIC values of 0.111% and 0.037%, respectively (Table 2). The composition, structure and the functional groups of essential oils play an important role in contributing to their antimicrobial activity (Holley and Patel, 2005). The high antimicrobial activity of rosemary has been correlated to its carnosic acid content (Moreno *et al*., 2006). The main component of the nanoparticled rosemary solubilisate extract used in this study was carnosic acid, containing a minimum of 40%. No previous studies have examined the antimicrobial effect of nanoparticled rosemary, but results generated in similar studies involving the use of non-nanoparticled rosemary application in cheese did not show the same effects as determined in this study. Gammariello *et al*. (2008) reported that rosemary was unacceptable as an active agent in inhibiting the spoilage microflora of Fior di Latte cheese. In our study, rosemary also had an extremely significant ($P \leq 0.001$) antimicrobial effect on *S. aureus* (mean MIC = 0.111%) and *B. cereus* (mean MIC = 0.037%), both Gram-positive bacteria (Table 2). Similarly, Shelef *et al*. (1980) examined rosemary against a range of Gram-positive and Gram-negative bacteria in growth media. Rosemary proved inhibitory, exhibiting the greatest activity against Gram-positive bacteria, which agrees with the results found in our study. On comparing rosemary extract with the sorbic acid or benzoic acid nanoparticled

solubilisates, it is clear that rosemary exhibited a greater inhibition range and at lower concentrations.

4.3.4 Examination of low molecular weight chitosan, medium molecular weight chitosan and acetic acid

Both low molecular weight chitosan and medium molecular weight chitosan demonstrated the widest range of antimicrobial activities by successfully inhibiting growth against all microbial cultures and were the only agents to exhibit action against the Gram-negative species assessed. In addition, both chitosans demonstrated the lowest overall MICs, with low molecular weight chitosan performing better than medium molecular weight chitosan, overall mean values of 0.0045% and 0.067%, respectively (Table 2). It has been postulated that different levels of antimicrobial activity may be experienced due to different molecular weights providing different mechanisms of inhibition. For example, low molecular weight chitosan can penetrate the bacterial cell, bind to DNA and subsequently inhibit RNA and protein synthesis (Hadwiger *et al*., 1985). The predominant mechanism of inhibition is an electrostatic interaction between chitosan and cell walls and cell membranes. It has been proposed that the positively charged amino groups of the glucosamine units create a polycationic structure, thereby interacting with negatively charged components in microbial cell membranes and altering their barrier properties, so preventing the entry of nutrients or causing the leakage of intracellular contents through the cellular membrane (Helander *et al*., 2001; Coma *et al*., 2003). Low molecular weight chitosan had a lower MIC than medium molecular weight chitosan with the exception of cottage cheese, for which medium molecular weight chitosan had an equal MIC. The only significant ($P \leq 0.05$) difference observed in MIC values

between both forms of chitosan was for Emmental-derived cultures (Table 4). Our findings were supported by those of Liu *et al*. (2006) who also showed that the antimicrobial activity of low molecular weight chitosan was greater than that of high molecular weight chitosan.

Chitosan, of both molecular weights, was generally more effective at inhibiting Gram-negative bacteria, which is in agreement with a study by Devlieghere *et al*. (2004). These authors also found that Gram-negative bacteria were more sensitive to chitosan, while the sensitivity of Gram-positive bacteria to chitosan was variable. However, most research has documented that chitosan has a stronger bactericidal effect on Gram-positive bacteria than on Gram-negative bacteria (No *et al*., 2002; Fernandez-Saiz *et al*., 2009). Zheng and Zhu (2003) suggested different mechanisms for the inhibition of Gram-positive and Gram-negative species. Chitosan can form a polymeric membrane on the cell surface of Gram-positive bacteria thus preventing nutrients from entering the cell. In Gram-negative bacteria, chitosan can permeate through the cell and disturb the physiological activities of the cell.

Acetic acid was used in the dissolution of chitosan. Like low molecular weight chitosan and medium molecular weight chitosan, it also exhibited an antimicrobial action on all culture samples and this effect was also significant. However, the overall mean MIC observed was 0.165%. Compared with the overall MIC values for chitosan, which were considerably lower, it is indicative that although acetic acid contributes to inhibition, the majority of antimicrobial action is as a consequence of the chitosan. This can be supported by observations from statistical analysis of acetic acid with either molecular weight of chitosan (Table 4). The differences reported support the efficacy of chitosan.

Table 4 Paired T-Test - Examination of Chitosan and Acetic acid.

Pairs		Difference	Significance
LMWC	MMWC	Emmental	*
		E.coli	ns
		P.fluorescens	ns
		S.aureus	ns
		B.cereus	ns
LMWC	AA	*E.coli*	*
		P.fluorescens	**
		S.aureus	*
		B.cereus	ns
MMWC	AA	Emmental	*
		P.fluorescens	*
		S.aureus	ns
		B.cereus	*

The table describes the statistical differences computed.

The level of significance is denoted by these asterisks;

* = $P \leq 0.05$ - 0.01 (significant), ** = $P \leq 0.01$ - 0.001

(highly significant), *** = $P \leq 0.001$ (extremely significant).

Means with the letters 'ns' are non-significant, $P > 0.05$.

4.4 CONCLUSION

Application of various antimicrobials to various microorganism including microbial cultures derived from cheese demonstrated a positive effect by inhibiting growth. The most persuasive nanoparticle was found to be rosemary extract. The other nanoparticled solubilisates investigated did not perform as expected; however, their greater solubility (sorbic acid, benzoic acid, curcumin) makes them more suitable for testing than their equivalent standard solutions. Non-nanoparticled chitosan, of low molecular weight and medium molecular weight, dissolved in an acetic acid medium, proved to be the most effective antimicrobial with the greatest inhibition range and the lowest MIC values. Future work in this area will focus on examining the combinations of antimicrobials with known abilities, to determine if a broader range of inhibition can be achieved at lower concentrations. This study found that rosemary nanoparticles were particularly effective at inhibiting Gram-positive bacteria and that Gram-negative bacteria were sensitive to low molecular weight chitosan. The potential of chitosan and rosemary to be used as active agents either singly or in combination, in packaging is great, especially in the area of cheese packaging, as both Emmental and cottage cheese microflora were very responsive to their action.

CHAPTER 5

Evaluation of the potential synergistic antimicrobial effects observed using combinations of nanoparticled and non-nanoparticled agents on cheese-derived microorganisms

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ABSTRACT

The objective of this study was to determine whether a combination of agents could produce a synergistic antimicrobial effect, by either targeting a greater spectrum of microorganisms or reducing the concentration of antimicrobial required to cause inhibition. Five agents (nanoparticled solubilisates – sorbic acid, benzoic acid and rosemary extract, and non-nanoparticled chitosans – of two different molecular weights) were selected based on promising antimicrobial activity and/or enhanced solubility. Combinations of these agents were examined against cultures derived from cheese, as well as selected Gram–positive and –negative species. The study found the top-performing antimicrobials contained chitosan and/or rosemary, individually or in combination. These findings encourage their use as active agents in cheese packaging.

5.1 INTRODUCTION

The driving force for the use of antimicrobial packaging for dairy foods, such as cheese, is due to the increase in demand for such products globally, with global consumers requiring the same standard of quality and safety as those purchasing these products on the domestic market. Exportation of cheese, like any other perishable product, is accompanied by many challenges. The problems imposed include increased exposure to fluctuating temperatures and humidities, increased handling, excessive distribution distances, and poor distribution and storage conditions. These factors can cause increased oxygen levels (Chapter 3) and changes to the physical and chemical characteristics of the cheese, including colour, texture, taste, oxidation, odour development, sweating, shape deformities, decrease in nutritional value and an increase in spoilage microorganisms; all of which can lead to a decrease in shelf-life and a compromised quality, providing a final product of an unacceptable standard.

The use of active packaging changes the condition of the packaged food. Active packaging extends the shelf-life, improves food safety or alters the sensory properties, whilst maintaining the quality of the packaged food (De Kruijf *et al*., 2002). Different preservatives have been employed in antimicrobial packaging over the years, with polysaccharides, essential oils derived from herbs and plants, organic acids and their salts, and bacteriocins most commonly associated with cheese preservation (Kasrazadeh and Genigeorgis, 1995; Scannell *et al*., 2000; Gammariello *et al*., 2008; Cerqueira *et al*., 2010; Hauser and Wunderlich, 2011). In addition, a number of studies have examined the effect of various combinations of antimicrobials on cheese to determine whether synergistic antimicrobial relationships between agents could be achieved (Sinigaglia *et al*., 2008; Fajardo *et al*., 2010;

Hanušová *et al*., 2010). The aim of utilising active agent combinations is to expand the antimicrobial spectrum reached, minimise toxicity, reduce concentration levels and obtain an overall synergistic antimicrobial activity (Song *et al*., 2003). However, many of these combinations to date have contained synthetic chemical agents, whereas the demand in active packaging for food applications is for natural antimicrobials. Additionally, there is an increased drive for the incorporation of nanotechnology into smart packaging design, as the area encompassing nano-based research is rapidly growing (Mangematin and Walsh, 2012).

The antimicrobial agents investigated in this study were selected based on results determined from Chapter 4. The criteria for this selection comprised a balance of promising antimicrobial activity and/or enhanced solubility. Sorbic acid and benzoic acid nanoparticled solubilisates were chosen due to their increased solubility over normal-sized sorbic and benzoic acid. A study by Cruz-Romero *et al*. (2013) demonstrated the considerable antimicrobial activity of nanoparticled sorbic and benzoic acid solubilisates relative to their non-nano-equivalents. Nanoparticled rosemary extract solubilisate showed a notable balance of enhanced solubility and an antimicrobial affinity towards cheese-derived cultures and Gram-positive bacteria (Chapter 4). To our knowledge, no other studies have explored the antimicrobial effect of nanoparticled rosemary or rosemary extract. Non-nanoparticled chitosan is well established as having many applications as an antimicrobial agent (Rabea *et al*., 2003; Aider, 2010). Previous work by No *et al*. (2002) and Zheng and Zhu (2003) have examined the influence of molecular weight on the degree of antimicrobial inhibition, but the impact of this characteristic on chitosan when used in combination with other antimicrobials has not been investigated as thoroughly.

Therefore, this study was undertaken to investigate the antimicrobial activity of nanoparticled benzoic acid, sorbic acid and rosemary extract solubilisates, and non-nanoparticled low molecular weight chitosan and medium molecular weight chitosan, when applied individually and in combination against cheese-derived cultures, as well as both Gram-negative and Gram-positive varieties.

5.2 MATERIALS AND METHODS

5.2.1 Materials and microbiological media

Aquanova AG (Darmstadt, Germany) supplied the four nanoparticled solubilisates (~30 nm) – 4% sorbic acid, 12% benzoic acid, 6% carnosolic acid (rosemary extract) and 4% sorbic acid/benzoic acid (1:1). Both chitosans, low molecular weight (50–190 kDa) and medium molecular weight (190–310 kDa), were sourced from Sigma–Aldrich, St. Louis, MO, USA. Acetic acid (Fisher Scientific UK Ltd., Leicestershire, UK) was used to improve the solubility of chitosan in water. Emmental and cottage cheese were both sourced locally. Tryptone soya agar (TSA) and Mueller–Hinton broth (MHB) were obtained from Oxoid Ltd., Basingstoke, Hampshire, UK. Minimum inhibition concentration (MIC) was measured using 96-well tissue culture microplates (Sarstedt, Inc., Newton, NC, USA).

5.2.2 Cultures and their growth conditions

The bacterial strains used for MIC testing including the Gram-negative species *Escherichia coli* (NCIMB 11943) and *Pseudomonas fluorescens* (NCIMB 9046) and the Gram-positive species *Staphylococcus aureus* (NCIMB 13062) and *Bacillus cereus* (NCIMB 9373), which were cultivated on TSA slants. Prior to MIC testing, the microbial cultures were regenerated twice from the TSA slants into a growth media, MHB, and incubated for 18 hr, at 30 °C for *B. cereus* and *P. fluorescens*, and at 37 °C for *S. aureus* and *E. coli*. General cheese cultures were derived from both Emmental and cottage cheese. Emmental culture preparation involved homogenising 10 g of Emmental with 90 ml of sterile MHB in a Colworth

Stomacher 400 (Seward Ltd., Worthing, UK). The homogenate (1 ml) was transferred into 10 ml MHB and incubated for 18 hr at 37 °C. Cottage cheese culture was prepared by swabbing the cottage cheese surface and transferring the swab into MHB (10 ml). The sample was then incubated for 18 hr at 37 °C.

5.2.3 Antimicrobial preparation

The antimicrobials selected included three nanoparticles – sorbic acid (SASB), benzoic acid (BASB) and rosemary extract (ROSE), and two non-nanoparticled chitosans – low molecular weight chitosan (LMWC) (50 000–190 000 Da) and medium molecular weight chitosan (MMWC) (190 000–310 000 Da). The nanoparticled solubilisates were standardised at a concentration level of 0.5% (w/v). Both solubilisates and sterile distilled water were preheated to 40 °C prior to mixing. Non-nanoparticled chitosan was prepared at 0.25% (w/v) in a 1% (v/v) acetic acid in a sterile distilled water solution at room temperature. Stock solutions were prepared for each of the levels of antimicrobials used. These five agents were input into the statistical program Statgraphics® Centurion XV (StatPoint, Inc., Warrenton, VA USA), which computed 32 different experimental mixtures. According to the mixtures computed via Statgraphics®, solutions from 1 to 33 were prepared (Table 1). Each solution was subjected to magnetic stirring to ensure homogeneity. In addition, another nanoparticled solubilisate – a mixture of sorbic acid and benzoic acid (SABASB) – was examined and labelled as solution 6.

Table 1

Antimicrobial mixtures, concentration breakdown and the total % concentration applied.

	Antimicrobial Mixtures	Concentration Breakdown	%
1	LMWC	0.25	0.25
2	MMWC	0.25	0.25
3	SASB	0.5	0.5
4	BASB	0.5	0.5
5	ROSE	0.5	0.5
6	SABASB	0.5	0.5
7	LMWC + MMWC	0.25 + 0.25	0.5
8	SASB + LMWC	0.5 + 0.25	0.75
9	SASB + MMWC	0.5 + 0.25	0.75
10	BASB + LMWC	0.5 + 0.25	0.75
11	BASB + MMWC	0.5 + 0.25	0.75
12	ROSE + LMWC	0.5 + 0.25	0.75
13	ROSE + MMWC	0.5 + 0.25	0.75
14	SASB + BASB	0.5 + 0.5	1
15	SASB + ROSE	0.5 + 0.5	1
16	BASB + ROSE	0.5 + 0.5	1
17	SASB + LMWC + MMWC	0.5 + 0.25 + 0.25	1
18	BASB + LMWC + MMWC	0.5 + 0.25 + 0.25	1
19	ROSE + LMWC + MMWC	0.5 + 0.25 + 0.25	1
20	SASB + BASB + ROSE + LMWC + MMWC	0.25 + 0.25 + 0.25 + 0.125 + 0.125	1
21	SASB + BASB + LMWC	0.5 + 0.5 + 0.25	1.25
22	SASB + BASB + MMWC	0.5 + 0.5 + 0.25	1.25
23	SASB + ROSE + LMWC	0.5 + 0.5 + 0.25	1.25
24	SASB + ROSE + MMWC	0.5 + 0.5 + 0.25	1.25
25	BASB + ROSE + LMWC	0.5 + 0.5 + 0.25	1.25
26	BASB + ROSE + MMWC	0.5 + 0.5 + 0.25	1.25
27	SASB + BASB + ROSE	0.5 + 0.5 + 0.5	1.5
28	SASB + BASB + LMWC + MMWC	0.5 + 0.5 + 0.25 + 0.25	1.5
29	SASB + ROSE + LMWC + MMWC	0.5 + 0.5 + 0.25 + 0.25	1.5
30	BASB + ROSE + LMWC + MMWC	0.5 + 0.5 + 0.25 + 0.25	1.5
31	SASB + BASB + ROSE + LMWC	0.5 + 0.5 + 0.5 + 0.25	1.75
32	SASB + BASB + ROSE + MMWC	0.5 + 0.5 + 0.5 + 0.25	1.75
33	SASB + BASB + ROSE + LMWC + MMWC	0.5 + 0.5 + 0.5 + 0.25 + 0.25	2

Antimicrobial abbreviations were assigned as follows: SASB - Sorbic Acid Solubilisate, BASB - Benzoic Acid Solubilisate, LMWC - Low Molecular Weight Chitosan, MMWC - Medium Molecular Weight Chitosan, SABASB - Sorbic Acid/Benzoic Acid Solubilisates.

5.2.4 Antimicrobial susceptibility assessment

Minimum inhibition concentration testing was used to determine the antimicrobial action of the prepared mixtures against various cultures through the microdilution method. This microdilution was executed via 96-well tissue culture microplates. Within the microplates, 100 µl of sterile MHB was pipetted into rows A to F, 1–12,

with an additional aliquot of 200 µl of MHB into the well H 12. Quantities of the antimicrobial mixture (150 µl) were pipetted into to row G, with row H 1–11 containing 200 µl of the test culture. Dilution was performed by transferring 50 µl of the antimicrobial from row G and mixing it into row F. Subsequently, 50 µl of the resultant mixture from row F was extracted and mixed into row E. This same action was repeated until row B, from which 50 µl was discarded, thus creating a threefold serial dilution. Row A contained no antimicrobial and was used as a positive growth control. Following dilution, each well from row A to G was inoculated with test culture (15 µl) from row H. Column 12 represented a no growth control as it contained no culture. The microplates were incubated for 18 hr, at 30 °C for *P. fluorescens* and *B. cereus*, and at 37 °C for *E. coli*, *S. aureus* and both Emmental- and cottage cheese-derived cultures. Turbidity was identified as an indication of growth, which was evaluated visually after incubation. Minimum inhibition concentration was defined as the lowest concentration of antimicrobial agent showing a complete growth inhibition of the microbial culture tested and expressed as a % (w/v).

5.2.5 Statistical analysis

The experiment was performed twice in triplicate. The total number of data points for each antimicrobial solution being six. The experimental data were analysed on SPSS Statistics 20 (IBM, Armonk, NY, USA). The mean and standard deviation for each antimicrobial mixture were calculated. ANOVA and Tukey's post hoc tests were used to determine the statistical significance between treatments within a test culture, and paired *t*-tests were used to determine the statistical significance between the means of related cultures (Cheese, Gram-negative, Gram-positive). The level of

significance was set at $P \leq 0.05–0.01$ (significant), $P \leq 0.01–0.001$ (highly significant) and $P \leq 0.001$ (extremely significant). Means with the letters ‘ns’ are non-significant, $P > 0.05$.

5.3 RESULTS AND DISCUSSION

The antimicrobial effects of the 33 combinations of antimicrobial agents assessed against microbial cultures using the microdilution assay are shown in Table 2 (unless otherwise stated). It can be seen from Table 2 that all treatments, with the exception of sorbic acid/benzoic acid solubilisate (SABASB), exerted overall antimicrobial effects. It was also determined that not all treatments demonstrated a complete antimicrobial effect on all cultures.

5.3.1 Assessment against cheese-derived cultures

In this study, the five most active antimicrobials against the microbial culture derived from cottage cheese were 0.25% MMWC (0.046%), 0.25% LMWC (0.053%), 0.5% ROSE (0.066%), 0.75% ROSE+LMWC (0.102%) and 0.5% LMWC+MMWC (0.111%), with no significant differences determined between them. The five best functioning antimicrobial treatments against the Emmental-derived culture were 0.25% LMWC (0.046%), 0.5% LMWC+MMWC (0.074%), 0.25% MMWC (0.083%), 0.75% ROSE+LMWC (0.111%) and 1% ROSE+LMWC+MMWC (0.148%) (MIC in brackets), again with no significant differences determined between them (Table 2). Chitosan and rosemary were the most effective antimicrobial agents assessed, with both substances previously reported to reduce bacterial counts on cheese (Coma *et al*., 2002; Mohamed *et al*., 2009). Although no significance was determined between antimicrobial treatments applied against cottage cheese- or Emmental-derived cultures individually, when the antimicrobial activities observed between both cheese culture types were compared, a significant difference in the effectiveness of treatments was found ($P < 0.05$) (Table 3).

Table 2

Mean minimum inhibition concentration (MIC - %, w/v) and standard deviation of the antimicrobial soultions against the various cultures. Values correspond to mean data for six test samples each, ± corresponds to the standard deviation.

	Antimicrobial Solution	Cottage Cheese	Emmental	*Escherichia coli*	*Pseudomonas fluorescens*	*Staphylococcus aureus*	*Bacillus cereus*	Total
1	LMWC 0.25%	**0.053 ± 0.035** [a]	**0.046 ± 0.029** [a]	**0.062 ± 0.034** [a]	**0.025 ± 0.008** [a]	**0.083 ± 0.000** [a]	**0.037 ± 0.023** [a]	**0.051 ± 0.030**
2	MMWC 0.25%	**0.046 ± 0.029** [a]	**0.083 ± 0.000** [a]	**0.065 ± 0.029** [a]	**0.046 ± 0.029** [a]	**0.083 ± 0.000** [a]	**0.046 ± 0.029** [a]	**0.062 ± 0.027**
3	SASB 0.5%	0.445 ± 0.136	0.500 ± 0.000	0.500 ± 0.000	0.500 ± 0.000	0.500 ± 0.000	0.500 ± 0.000	0.491 ± 0.056
4	BASB 0.5%	0.445 ± 0.136	0.500 ± 0.000	0.500 ± 0.000	0.500 ± 0.000	0.500 ± 0.000	0.445 ± 0.136	0.482 ± 0.077
5	ROSE 0.5%	**0.066 ± 0.053** [a]	0.370 ± 0.204	0.500 ± 0.000	0.500 ± 0.000	0.315 ± 0.207	**0.037 ± 0.020** [a]	0.298 ± 0.220
6	SABASB 0.5%	0.500 ± 0.000	0.500 ± 0.000	0.500 ± 0.000	0.500 ± 0.000	0.500 ± 0.000	0.500 ± 0.000	0.500 ± 0.000
7	LMWC/MMWC 0.5%	**0.111 ± 0.061** [a]	**0.074 ± 0.045** [a]	**0.130 ± 0.057** [a]	**0.056 ± 0.000** [a]	**0.167 ± 0.000** [a]	**0.093 ± 0.057** [a]	**0.105 ± 0.056**
8	SASB/LMWC 0.75%	0.210 ± 0.098	0.306 ± 0.228	**0.222 ± 0.068** [b]	0.250 ± 0.000	0.500 ± 0.274	0.250 ± 0.000	0.290 ± 0.174
9	SASB/MMWC 0.75%	0.222 ± 0.068	0.250 ± 0.000	0.250 ± 0.000	0.222 ± 0.068	0.500 ± 0.274	0.250 ± 0.000	0.282 ± 0.148
10	BASB/LMWC 0.75%	0.222 ± 0.068	0.222 ± 0.068	0.250 ± 0.000	0.222 ± 0.068	0.500 ± 0.274	0.167 ± 0.091	0.264 ± 0.161
11	BASB/MMWC 0.75%	0.185 ± 0.102	0.250 ± 0.000	0.250 ± 0.000	0.250 ± 0.000	**0.222 ± 0.068** [a]	0.250 ± 0.000	0.235 ± 0.053
12	ROSE/LMWC 0.75%	**0.102 ± 0.076** [a]	**0.111 ± 0.068** [a]	0.250 ± 0.000	0.222 ± 0.068	0.472 ± 0.310	0.139 ± 0.086	**0.216 ± 0.183**
13	ROSE/MMWC 0.75%	0.157 ± 0.103	0.250 ± 0.000	0.250 ± 0.000	0.250 ± 0.000	0.472 ± 0.310	**0.065 ± 0.029** [a]	0.241 ± 0.176
14	SASB/BASB 1%	0.889 ± 0.272	1.000 ± 0.000	1.000 ± 0.000	1.000 ± 0.000	1.000 ± 0.000	1.000 ± 0.000	0.982 ± 0.111
15	SASB/ROSE 1%	0.617 ± 0.433	1.000 ± 0.000	1.000 ± 0.000	1.000 ± 0.000	0.667 ± 0.365	0.210 ± 0.138	0.749 ± 0.367
16	BASB/ROSE 1%	0.506 ± 0.399	1.000 ± 0.000	1.000 ± 0.000	1.000 ± 0.000	0.519 ± 0.383	0.185 ± 0.115	0.702 ± 0.387
17	SASB/LMWC/MMWC 1%	0.259 ± 0.115	0.296 ± 0.091	0.259 ± 0.115	**0.222 ± 0.122** [c]	0.333 ± 0.000	0.333 ± 0.000	0.284 ± 0.094
18	BASB/LMWC/MMWC 1%	0.185 ± 0.115	0.259 ± 0.115	**0.222 ± 0.122** [b]	0.259 ± 0.115	0.333 ± 0.000	0.185 ± 0.115	0.241 ± 0.111
19	ROSE/LMWC/MMWC 1%	0.185 ± 0.115	**0.148 ± 0.091** [a]	0.284 ± 0.121	**0.111 ± 0.000** [b]	**0.296 ± 0.091** [a]	0.185 ± 0.115	**0.202 ± 0.114**
20	SASB/BASB/ROSE/LMWC/MMWC 1%	0.259 ± 0.115	0.333 ± 0.000	0.333 ± 0.000	0.333 ± 0.000	0.667 ± 0.365	0.333 ± 0.000	0.377 ± 0.198
21	SASB/BASB/LMWC 1.25%	0.370 ± 0.113	0.417 ± 0.000	0.417 ± 0.000	0.417 ± 0.000	1.250 ± 0.000	0.417 ± 0.000	0.548 ± 0.322
22	SASB/BASB/MMWC 1.25%	0.509 ± 0.380	0.556 ± 0.340	0.417 ± 0.000	0.417 ± 0.000	1.111 ± 0.340	0.417 ± 0.000	0.571 ± 0.342
23	SASB/ROSE/LMWC 1.25%	0.401 ± 0.444	0.417 ± 0.000	0.417 ± 0.000	0.417 ± 0.000	0.833 ± 0.456	0.216 ± 0.160	0.450 ± 0.312
24	SASB/ROSE/MMWC 1.25%	0.324 ± 0.144	0.695 ± 0.430	0.417 ± 0.000	0.417 ± 0.000	0.833 ± 0.456	0.278 ± 0.152	0.494 ± 0.323
25	BASB/ROSE/LMWC 1.25%	0.355 ± 0.151	0.463 ± 0.409	0.417 ± 0.000	0.417 ± 0.000	0.648 ± 0.478	0.232 ± 0.144	0.422 ± 0.281
26	BASB/ROSE/MMWC 1.25%	0.324 ± 0.144	0.417 ± 0.000	0.417 ± 0.000	0.417 ± 0.000	0.833 ± 0.456	0.185 ± 0.113	0.432 ± 0.273
27	SASB/BASB/ROSE 1.5%	0.759 ± 0.599	1.500 ± 0.000	1.500 ± 0.000	1.500 ± 0.000	1.333 ± 0.408	0.445 ± 0.136	1.173 ± 0.507
28	SASB/BASB/LMWC/MMWC 1.5%	0.445 ± 0.136	0.500 ± 0.000	0.445 ± 0.136	0.500 ± 0.000	0.667 ± 0.408	0.500 ± 0.000	0.509 ± 0.187
29	SASB/ROSE/LMWC/MMWC 1.5%	0.370 ± 0.204	0.333 ± 0.183	0.389 ± 0.172	0.445 ± 0.136	0.833 ± 0.516	0.167 ± 0.000	0.423 ± 0.313
30	BASB/ROSE/LMWC/MMWC 1.5%	0.313 ± 0.209	0.556 ± 0.491	0.500 ± 0.000	0.445 ± 0.136	1.000 ± 0.548	0.333 ± 0.183	0.524 ± 0.381
31	SASB/BASB/ROSE/LMWC 1.75%	1.037 ± 0.794	0.583 ± 0.000	0.583 ± 0.000	0.583 ± 0.000	1.556 ± 0.476	0.583 ± 0.000	0.821 ± 0.512
32	SASB/BASB/ROSE/MMWC 1.75%	0.648 ± 0.573	0.583 ± 0.000	0.583 ± 0.000	0.583 ± 0.000	1.361 ± 0.603	0.519 ± 0.159	0.713 ± 0.436
33	SASB/BASB/ROSE/LMWC/MMWC 2%	0.889 ± 0.878	0.667 ± 0.000	0.667 ± 0.000	0.667 ± 0.000	1.111 ± 0.689	0.667 ± 0.000	0.778 ± 0.455
	Total MIC for each culture	0.376 ± 0.377	0.460 ± 0.342	0.454 ± 0.301	0.445 ± 0.311	0.667 ± 0.489	0.308 ± 0.221	

Data in the columns highlighted in bold are the top five treatments for that culture.

Only the differences between the top five treatments for each test culture are shown. Letters (a, b, c) within columns represent statistical differences ($P \leq 0.05$) between the treatments.

Table 3

Paired Samples Test - Difference between pairs.

Pairs		Significance
Cottage Cheese	Emmental	*
E. coli	*P. fluorescens*	ns
S. aureus	*B. cereus*	***

The level of significance is denoted by these asterisks;
* = P < 0.05 - 0.01 (significant), ** = P < 0.01 - 0.001 (highly significant), *** = P < 0.001 (extremely significant).
Means with the letters 'ns' are non-significant, P > 0.05.

Emmental microflora showed a greater resistance than cottage cheese to the treatments used. In total, seven treatments produced no antimicrobial effect against the Emmental-derived culture, whereas only one treatment (SABASB) failed to produce an antimicrobial effect against the cottage cheese-derived culture. From the MICs generated, it can be seen that the cottage cheese-derived culture also presented a lower MIC, which implies that cottage cheese-derived culture was more sensitive to the treatments applied. This is an interesting finding as it shows differences in inherent resistances to chemical treatments by cultures derived from different cheese products. Such differences have been reported previously when chemical treatments have been applied to cheese products, and the reasons proposed for variations in antimicrobial efficacy have been attributed to the physical structure and composition of the cheese product in question. It has been proposed that components present within the cheese may provide a level of protection which might prevent interaction between the antimicrobial substance and the target microorganisms. Selim (2011) suggested that differences in cheese morphology and composition, in particular fat, protein and level of water content, could be responsible for a diminished level of antimicrobial activity. A high fat content can impair the capacity of an antimicrobial to reduce a microbial population (Ribeiro *et al.*, 2013). Specifically for cheese,

Smith-Palmer *et al*. (2001) found a reduced antimicrobial activity in higher fat cheeses. The fat present can form a protective barrier around the bacteria, and additionally, the antimicrobial agent could dissolve into the lipid fraction which decreases the concentration of antimicrobial available, thereby reducing its capacity to act against bacteria in the aqueous phase (Mejlholm and Dalgaard, 2002; Patel *et al*., 2005). Emmental has a higher total fat content (29.7 g) than cottage cheese (4.3 g) (Food Standards Agency, 2002). The increased lipid levels may explain the lower inhibition observed for Emmental. Similarly, Emmental has a much lower water content than cottage cheese. Low water content may impair the movement of the antimicrobial agents to the active site of the bacterial cell (Smith-Palmer *et al*., 2001). These factors may explain why chemical treatments and their activities may be diminished when applied to cheese products, and the results presented here show clearly that inherent differences within cheese-derived cultures will present their own challenges and resistances when chemical treatments are applied.

5.3.2 Assessment against Gram-negative species

The results produced from the antimicrobial testing of Gram-negative bacteria show remarkable similarities between the inhibition of *E. coli* and *P. fluorescens* (Table 2). The overall MICs for *E. coli* and *P. fluorescens* are relatively comparable at 0.454% and 0.445%, respectively, as are the three most effective working treatments – LMWC, MMWC and LMWC+MMWC. Tsai and Su (1999) and Coma *et al*. (2003) have both demonstrated the inhibitory effect of chitosan on *E. coli* and *Pseudomonas* species, respectively. BASB+LMWC+MMWC (0.222%) and SASB+LMWC (0.222%) treatments make up the five most effective treatments

against *E. coli*, suggesting that organic acids provide a marginal antimicrobial effect on *E. coli*. Within the five most effective treatments against *E. coli*, LMWC, MMWC, and LMWC+MMWC were found to be significantly different ($P < 0.05$) from BASB+LMWC+MMWC and SASB+LMWC (Table 2). The remaining top-performing antimicrobial agents against *P. fluorescens* included ROSE+LMWC+MMWC (0.111%) and SASB+LMWC+MMWC (0.222%), with SASB+LMWC+MMWC being significantly different ($P < 0.05$) from the other treatments (LMWC, MMWC, LMWC+MMWC and ROSE+LMWC+MMWC) (Table 2). A total of 10 and 11 treatments demonstrated antimicrobial activity at a concentration of 0.25% or below against *E. coli* and *P. fluorescens*, respectively. Nanoparticled rosemary extract demonstrates an acuteness for *P. fluorescens*, which it does not appear to possess for *E. coli*. An improvement in Gram-negative inhibition may be achievable, if rosemary was to be added at a higher concentration. Mendoza-Yepes *et al.* (1997) found that increased levels of essential oils were required to inhibit Gram negative compared to the levels needed to inhibit the Gram-positive range of bacteria present.

However, the strongest antimicrobial effects exerted on Gram-negative bacteria in this study were seen for chitosan-based treatments. The antimicrobial mechanism associated with chitosan is attributed to chitosan's ability bind to the outer membrane of the bacterial cell and subsequently disrupt barrier function (Helander *et al.*, 2001). Even though chitosan provided the greatest antimicrobial effect for both microorganisms, *P. fluorescens* had noticeably lower MIC values. This could be due to the *E. coli* possessing an early warning defence mechanism against antimicrobial attack (Rowbury, 2001). In any case, when MIC data for *E. coli* and *P. fluorescens* were compared and no significant differences were found (Table 3).

5.3.3 Assessment against Gram-positive species

Unlike the treatment similarities observed for Gram-negative bacteria, there was a stark contrast in results between *S. aureus* and *B. cereus* (Table 2). *Staphylococcus aureus* endured the highest overall MIC (0.667%) amongst all samples tested, whereas *B. cereus* experienced the lowest MIC (0.308%). The five most effective antimicrobial treatments for both Gram-positive bacteria assessed were similar (MIC in brackets) for both *B. cereus* – 0.5% ROSE (0.037%), 0.25% LMWC (0.037%), 0.25% MMWC (0.046%), 0.75% ROSE+MMWC (0.065%) and 0.5% LMWC+MMWC (0.093%) and *S. aureus* – 0.25% LMWC (0.083%), 0.25% MMWC (0.083%), 0.5% LMWC+MMWC (0.167%), 0.75% BASB+MMWC (0.222%) and 1% ROSE+LMWC+MMWC (0.296%), with no significant differences between these treatments (Table 2). However, as can be readily observed, the treatment levels required to deliver antimicrobial effects were very different ($P < 0.001$, Table 3). For *B. cereus*, a total of 30 active antimicrobial combinations were evident from screening; 18 of which had a MIC of less than 0.250%, with only SASB, SASB+BASB and SABASB proving to be non-active treatments. Conversely, 28 treatments had an antibacterial effect on *S. aureus*; however, only four of these treatments were effective at a concentration of less than 0.25%. Generally, Gram-positive bacteria are considered less resistant to antimicrobial substances than Gram-negative bacteria as they do not possess an outer membrane. However, certain Gram-positive microbes have been known to develop a protective response to compensate for the absence of this outer cell membrane. Staphylococci can illicit efficient mechanisms to neutralise antimicrobials (Lowy, 2003). For example, *S. aureus* has been known to use intercellular communication to induce virulence factors (Sifri, 2008). However, in this study, *S. aureus* tolerance to the

antimicrobials was most likely due to the natural variance within the microbe assessed rather than an actual stable resistance.

Bacillus cereus was the only microbe tested which showed sensitivity to an active antimicrobial treatment which did not possess chitosan as part of the treatment, BASB + ROSE (MIC – 0.185%) and SASB + ROSE (MIC – 0.210%). Another unique point with respect to the control of *B. cereus* was that the nanoparticled rosemary extract performed just as strongly as chitosan in treatments. Ivanovic *et al*. (2012) also determined that *B. cereus* and other *Bacillus* species were very susceptible to rosemary compared to other bacteria tested. Rosemary extract also impacted on *S. aureus*, but at a higher MIC level (0.315%). Campo *et al*. (2000) examined the antimicrobial effect of a commercial rosemary extract and, similar to our findings, found that much lower concentrations of rosemary were needed to inhibit *B. cereus* (0.06%) compared to *S. aureus* (0.5%).

5.3.4 Overall activity and synergism observations

Overall, the five best performing antimicrobial treatments were determined to be 0.25% LMWC, 0.25% MMWC, 0.5% LMWC+MMWC, 0.75% ROSE+LMWC and 1% ROSE+LMWC+MMWC. They had MICs (%) of 0.051, 0.062, 0.105, 0.202 and 0.216, respectively. This correlates with Chapter 4, which showed that LMWC, MMWC and nanoparticled rosemary extract all showed the greatest antimicrobial activities of the agents assessed. Chitosan is evidently the most effective broad-spectrum antimicrobial in this study due to its low MIC levels and, as evidenced by its presence in all of the five most effective active treatments, used either on its own or in combination. Chitosan of a lower molecular weight performed slightly better

than medium molecular weight chitosan, which is in agreement with our previous work (Chapter 4), but in contrast to the findings reported by Shin *et al*. (2001) who found that an increase in bacterial reduction as the molecular weight of chitosan increased. Overall, LMWC and MMWC functioned more effectively as antimicrobial substances when used on their own than when used in combination treatments. The nanoparticled rosemary extract itself exerted a moderate antimicrobial activity, working particularly well for both cheese-derived cultures and Gram-positive cultures. The organic acid solubilisates demonstrated only a marginal effect. Of the two organic acids tested, BASB (MIC = 0.482) performed better than SASB (MIC = 0.491). Da Rocha *et al*. (2014) showed similar results for both organic acids, although not in nanoparticulate form, against Gram-positive and Gram-negative bacteria following incorporation into packaging films.

Although it was hoped that stronger synergistic effects would be achieved between the agents assessed, a commensal influence was more evident. No combination treatment attained the same antimicrobial effectiveness as that produced by a single antimicrobial treatment. Gutierrez *et al*. (2008) also reported that various chemical combinations assessed in their study showed no synergism, but resulted in many additive, and some indifferent patterns. Park *et al*. (2004) suggested that chitosan has great compatibility with other antimicrobials due to its chemical structure. Studies have previously demonstrated that chitosan, when used in combination with other substances, has the capacity to enhance greater antimicrobial activity than either agent applied individually (Duan *et al*. 2007; Moreira *et al*., 2011). In general, the antimicrobial effects of the chitosan combinations, particularly those with rosemary, proved stronger than the chitosan–organic acid combinations. This has also been seen when chitosan was used in combination with garlic oil and potassium sorbate.

The activity of chitosan was substantially improved using the essential oil, but a reduced action was reported when chitosan was combined with the organic acid salt (Pranoto *et al*., 2005). The reduction in antimicrobial activity observed when chitosan and an organic acid are used in combination may be due to the decreased ability of chitosan to interact with the bacterial membrane (Vásconez *et al*., 2009).

Gutierrez *et al*. (2008) suggested that agents with a similar composition and structure may not provide synergistic effects. Although rosemary and organic acids do not have similar chemical compositions, nanoparticled solubilisates have related encapsulated micellular structures. Equally, LMWC and MMWC have similar structures and when used together in different combinations, they provided antimicrobial action but none of these combinations were as antimicrobially effective as either form of chitosan applied individually. Conversely, this could also explain why combinations of chitosan and solubilisates had an additive effect, owing to the different physical and chemical structures associated with these substances. In addition to chemistry and structure affecting efficacy, potency can also be affected by environmental conditions. Adjusting pH may be key to achieving synergism with solubilisates in the future. The use of a dispersing agent could enhance the contact of solubilisates with the microbial cells, especially in foods with a high fat content, such as Emmental cheese (Smith-Palmer *et al*., 2001). Additionally, the incorporation of natural chelators or enzymes could be used to disrupt the membrane of Gram-negative bacteria.

5.4 CONCLUSION

Chitosan, of low and medium molecular weight, and nanoparticled rosemary extract provided the most interesting and effective inhibition across all cultures examined. Overall, chitosan was the best performing antimicrobial of all screened agents, providing strong results when used singly or in combination, with low molecular weight chitosan functioning slightly better than medium molecular weight chitosan. Rosemary appeared to be more antimicrobially selective in its inhibition behaviour, providing a favourable effect against cheese-derived cultures and Gram-positive bacteria. No treatment combination proved to be synergistic. Lowering pH or incorporating membrane perturbing substances could be employed to improve solubilisate activity. Future work will concentrate on the incorporation of chitosan and/or nanoparticled rosemary extract treatments into packaging and applying the treated packaging to cheese products.

CHAPTER 6

Preparation of low- and medium-molecular weight chitosan nanoparticles and their antimicrobial evaluation against a panel of microorganisms, including cheese-derived cultures

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ABSTRACT

This study employed the technique of ionic gelation in the manufacture of low- and medium-molecular weight chitosan nanoparticles. Nanoparticles were characterised (size, size distribution, surface charge and morphology) and their antimicrobial activity assessed against cheese-derived cultures, as well as a select panel of Gram-positive and Gram-negative microorganisms. Antimicrobial activity was determined by the minimum inhibition concentration (MIC) via the micro dilution method using 96-well microplates. Synthesised particles were small-sized, with a moderate size distribution and positive zeta potential. Generated nanoparticles exhibited successful solubility in both water and acetic acid. Acidic nanosuspensions demonstrated greater microbial reduction than water-based nanoparticles, with no difference in activity observed between molecular weights. Cheese-derived cultures were effectively controlled, and Gram-negative species were more susceptible than Gram-positive species to the action of nanoparticles in acetic acid. Nanoparticles suspended in an acidic-based medium show promise as antimicrobial agents, particularly for use with cheese products.

6.1 INTRODUCTION

Chitosan is a polysaccharide derived via deacetylation from chitin. Chitin is naturally occurring and abundantly available as it is commonly found in the structural components of many invertebrates and in the cell walls of most fungi and some algae (Wang *et al*., 2004). Chitosan is considered to be an incredibly versatile polymer due to its chemical, physical and functional characteristics. These advantageous properties include its cationic nature, biodegradability, good adsorption capacity, biocompatibility, permeability-enhancing effect, film-forming capabilities, adhesive characteristics and many more, whilst being considered safe and cost-effective (Agnihotri *et al*., 2004; Fan *et al*., 2012). In particular, chitosan possesses a wide spectrum of inhibition against bacterial and fungal species, with antimicrobial activity being heavily dependent on molecular weight (Kong *et al*., 2008). However, chitosan is insoluble in water (Kim *et al*., 2006), which limits its utilisation in many applications and is often dissolved in an acidic medium to achieve potential purpose. Despite its solubility shortcomings, chitosan has been applied to many industries and fields, such as cosmetics, biotechnology, pharmaceutics, medical, water engineering, food and nutrition, photography, opthalmology, paper technology and others (Kumar, 2000).

Nanoparticled applications are becoming increasingly popular due to the additional functionalities imparted when microparticles are converted to nanoparticles. The advantages of utilising nanoparticles include improved dissolution and suspension stability, increased activity and permeability, higher loading capacity and availability, and an influence on sensorial characteristics (Horiba Instruments Inc., 2012; Malvern Instruments Ltd., 2012; Gokce *et al.,* 2014). Different methods of manufacture are available for synthesising chitosan nanoparticles. These include

coacervation/precipitation, emulsion by crosslinking or coalescence, inverse/reverse micelles, spray-drying, and others, but ionic gelation is often favoured as it is a simple, mild, and controllable process that can be conducted at a low-cost (Cota-Arriola *et al*., 2013). The formation of chitosan nanoparticles via the ionic gelation technique is based on the establishment of several electrostatic interactions between oppositely charged polymers – a polycationic chitosan and a polyanion, usually sodium tripolyphosphate (TPP) (Aktaş *et al*., 2005). Chitosan nanoparticles have been employed most prevalently as a carrier material or delivery system for proteins, drugs, vaccines and/or DNA, predominately in the area of pharmaceutics (Liu *et al*., 2007).

Although there is a great deal of literature on the characterisation of nanoparticled chitosan and some research into the antimicrobial activity of these nanoparticles, no investigations to our knowledge have reported the assessment of antimicrobial control of chitosan nanoparticles on food-derived cultures. Therefore, this research aims to manufacture low- and medium-molecular weight chitosan nanoparticles via ionic gelation and examine their antimicrobial effect against cheese-derived cultures, as well as various Gram-positive and Gram-negative species.

6.2 MATERIALS AND METHODS

6.2.1 Materials

Chitosan, of two molecular weights, were obtained from Sigma-Aldrich (St. Louis, MO, USA) and used as received. Low molecular weight chitosan (LMWC) has a molecular weight of 50-190 kDa, deacetylation degree of ≥ 75%, and a viscosity of 20-300 cP. Medium molecular weight chitosan (MMWC) has a molecular weight of 190-310 kDa, deacetylation degree of 75-85%, and a viscosity of 200-800 cP. Sodium tripolyphosphate (TPP), glacial acetic acid and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich (St. Louis, MO, USA), Fisher Scientific UK Ltd. (Leicestershire, UK), and KB Scientific (Cork, Ireland), respectively. Ultrapure water (PURELAB Option-Q, Elga, UK) was used throughout the study. Tryptone Soya Agar (TSA) and Mueller-Hinton Broth (MHB) were obtained from Oxoid Ltd. (Basingstoke, Hampshire, UK), and used as growth media. *E.coli* (NCIMB 11943), *B.cereus* (NCIMB 9373), *S.aureus* (NCIMB 13062), *P.fluorescens* (NCIMB 9046) were maintained on TSA slants until use at 4 °C. Emmental and Cottage cheese were both sourced locally. Minimum Inhibition Concentration (MIC) was measured using 96-well tissue culture microplates (Sarstedt, Inc., Newton, NC, USA).

6.2.2 Preparation of chitosan nanoparticles

Chitosan was prepared at 0.3% (w/v) in 1% (v/v) acetic acid aqueous solution and stirred overnight. The pH of the solution was adjusted to 4.6 using 10 N NaOH. TPP was dissolved in ultrapure water at a concentration of 0.3% w/v. At room temperature, the cross-linking of chitosan with TPP at a ratio of 5:1 was performed

through stirring at 800 rpm. TPP was added to the chitosan in a drop-wise manner via a Dose It P910 peristaltic pump (Integra Biosciences AG, Zizers, Switzerland) at a flow rate of 4.0 ml/min. Following the addition of TPP, an opalescent solution was obtained. According to Li *et al*. (2003), this is a visual indication of nanoparticle synthesis. The solution was stirred for a further 20 min, and sonicated at 80% amplitude. The resulting chitosan-TPP solution was subjected to centrifugation (12000 *g* for 15 min) to extract the nanoparticles from the suspension. Supernatant was discarded and the precipitate was redispersed in water by stirring. The solution was subsequently sonicated again and the centrifugal step repeated. The nanoparticles were then collected and the characterisation and antimicrobial properties were assessed.

6.2.3 Nanoparticle characterisation

The nanoparticles characterised were prepared fresh and analysed at a concentration of 0.25% (w/v) dispersed in both ultrapure water and in an aqueous acetic acid solution (1% v/v).

- *Mean particle size, polydispersity index (PDI) and zeta potential*

These measurements were acquired using a Zetasizer Nano ZS (Malvern, Worcestershire, UK). The analysis was performed at 25 °C, the dispersant refractive index and viscosity were defined as 1.33 and 0.8872 cP, respectively, and the material refractive index was set at 1.52. Particle size (d.nm – diameter) and PDI measurements were performed at 173° backscatter angle. The hydrodynamic

diameter is a measurement of particle size when particles are in an aqueous dispersion, and it is a more appropriate assessment than microscopy determinations (dehydrated nanoparticles) as particles are ultimately used in a dispersion on application. However, hydrodynamic diameter values are typically larger than microscopy estimations due to the swelling properties and adhesive nature of chitosan nanoparticles in liquid suspensions (Gokce *et al.* 2014). Zeta potential samples were evaluated in automatic mode. Each nanoparticle dispersion was measured in triplicate and reported as the mean ± standard deviation.

- *Morphology*

Chitosan nanoparticles were visualized using an Atomic Force Microscope (AFM) Park XE-100 (Suwon, Korea). A droplet of the nanoparticle suspension was deposited onto a silicon substrate and dried at room temperature. After drying, the samples were analysed in non-contact mode using non-contact high resolution micro-cantilever probe tips. Phase images of 4 μm^2 were obtained at an amplitude of 1.6-1.65 µm and resonance frequency of 325-335 MHz.

6.2.4 Antimicrobial activity assessment

- *Microbial growth conditions*

General mixed cheese cultures derived from Emmental and cottage cheese and a selection of Gram-positive and Gram-negative species were used to determine the antimicrobial activity of the chitosan nanoparticled solutions. Emmental cheese (10 g) was homogenised with 90 mls of sterile growth media (MHB) in a Colworth

Stomacher 400 (Seward Ltd., Worthing, UK), with 1 ml of the homogenate transferred into MHB. Cottage cheese culture was prepared by swabbing the cheese surface and transferring the swab into 10 ml MHB. Cheese cultures were incubated at 37 °C for 18 hr, followed by dilution into MHB to obtain 1.3 x 10^6 CFU/ml and 7.2 x 10^7 CFU/ml for Emmental and cottage cheese, respectively.

The bacterial species – *Escherichia coli*, *Pseudomonas fluorescens*, *Staphylococcus aureus*, and *Bacillus cereus*, were maintained on TSA slants at 4 °C. These strains were activated by inoculating into MHB and incubating for 18 hr, at 30 °C (*Pseudomonas fluorescens* and *Bacillus cereus*), and at 37 °C (*Escherichia coli* and *Staphylococcus aureus*). This generation was repeated to achieve an inoculum of 10^8-10^9 CFU/ml.

- *Preparation of chitosan nanoparticle solutions for antimicrobial assessment*

As prepared for characterisation, LMWC nanoparticles and MMWC nanoparticles were dispersed at 0.25 % (w/v) in both ultrapure water and in 1 % (v/v) acetic acid solution. Additionally, the pH of these solutions was evaluated.

- *Determination of MIC*

The antimicrobial action of the nanoparticled chitosan solutions was evaluated by determining the MIC using the micro-dilution method and 96-well tissue culture microplates. Within the microplates, sterile MHB (100 µl) was pipetted into rows A to F, 1-12, with an additional 200 µl of MHB inserted into H 12. The rest of row H (1-11), contained 200 µl of the test culture. In row G, 150 µl of 0.25% nanoparticled

chitosan solution was dispensed into the wells. A three-fold serial dilution was achieved by transferring 50 µl of the chitosan nanoparticle solution from row G and mixed into row F. The resultant mixture (50 µl) from row F was extracted and mixed into row E. This transformation was repeated to row B, from which 50 µl was discarded. Row A contained no nanoparticles and was used as a positive growth control. Following this serial dilution, rows A to G were inoculated with 15 µl from row H (test culture). Column 12 contained no culture and represented a no growth control. The microplates were incubated at 30 °C for *Pseudomonas fluorescens* and *Bacillus cereus*, and at 37 °C for *Escherichia coli*, *Staphylococcus aureus*, and both cheese-derived cultures. Following the 18 hr incubation period, turbidity was identified as an indication of growth, which was evaluated visually; the lowest concentration of chitosan solution demonstrating a complete growth inhibition being considered to be the MIC (%, w/v).

6.2.5 Statistical analysis

The antimicrobial assessment was performed twice in triplicate. The total number of MIC data points for each chitosan nanoparticle solution was six, with the MIC presented as mean ± standard deviation. Experimental data was analysed on SPSS Statistics 20 (IBM, Armonk, NY, USA). One-way ANOVA and Tukey's Post-Hoc tests were applied to determine statistical significance between treatments. *P* values ≤ 0.05 were considered to be statistically significant.

6.3 RESULTS AND DISCUSSION

The sediment post-centrifugation was white with a gel consistency. These nanoparticles (0.25%) were easily redispersed in ultrapure water or in a 1% acetic acid solution to achieve a transparent appearance.

6.3.1 Size, polydispersity index, zeta potential and morphology of chitosan nanoparticles

The mean particle size, polydispersity index and zeta potential of the manufactured chitosan nanoparticles is shown (Table 1). The mean particle size of the chitosan nanoparticle solutions was found to be 132 nm, 152 nm, 157 nm and 202 nm for MMWC + acetic acid, LMWC + water, MMWC + water and LMWC + acetic acid, respectively. Despite not being less than 100 nm, previous investigations have referred to those particles as nanoparticles, and whilst they are not technically 'nano' by definition, they are in the submicron range (nm) and will be referred to as nanoparticles. It has been reported previously by Wu *et al*. (2005) that smaller nanoparticles are produced when chitosan of a low molecular weight is used, however, the data generated from this study suggests that nanoparticles had a similar size, irrespective of molecular weight or dispersing medium.

Table 1

Characterisation of manufactured chitosan particles

	Particle Size (nm)	Polydispersity Index	Zeta Potential (mV)
LMWC + Water	152 ± 28.57	0.465 ± 0.00	54.4 ± 1.80
LMWC + 1% Acetic acid	202 ± 02.11	0.397 ± 0.04	61.8 ± 1.89
MMWC + Water	157 ± 22.77	0.516 ± 0.08	44.7 ± 1.68
MMWC + 1% Acetic acid	132 ± 06.70	0.560 ± 0.02	59.4 ± 1.68

LMWC - Low Molecular Weight Chitosan, MMWC - Medium Molecular Weight Chitosan

The polydispersity index (PDI) is a value which describes the size distribution of a sample. A PDI which is equal to 1 expresses that the sample has a very broad and variable size distribution. All nanoparticled solutions measured showed PDI values of 0.560 or less (Table 1), signifying an intermediate size profile. This indicates that agglomeration may have occurred, or more likely that dust particles are present. Filtering the aqueous medium prior to measurement could eliminate any dust particles present which may aid in reducing particle size and PDI values. LMWC nanoparticles demonstrated a narrower distribution compared to MMWC nanoparticles, which is consistent with results obtained by Rampino *et al*. (2013), who reported that the larger polymer chains associated with higher molecular weights contributed to a wide variety in particle size.

The zeta potential is a measure of the electrostatic repulsion between particles in an aqueous solution, and as a result it is a critical parameter in determining nanosuspension stability (Müller *et al*., 2001). As shown in Table 1, the zeta potential of LMWC in acetic acid (61.8 mV) was the highest, followed by MMWC in acetic acid (59.4 mV), LMWC in ultrapure water (54.4 mV), and MMWC in ultrapure water (44.7 mV); all of which exhibited a positive charge. Nanoparticles with a zeta potential greater than ± 30 mV are considered stable as there is enough repulsive force present to prevent aggregation between particles (Mohanraj and Chen, 2007). However, despite all the nanosuspensions exceeding the 30 mV threshold, both LMWC and MMWC in ultrapure water had lower zeta potentials than those dispersed in 1% acetic acid solutions, which not only suggests these particles have a lower stability, but that the dispersing medium contributes to the zeta potential magnitude. Mao *et al*. (2001) have previously shown that pH affects zeta potential, with a higher pH deriving a lower and/or negative zeta potential.

Nanoparticles dissolved in just water alone have a much higher pH value (Table 2), and according to Lavertu *et al*. (2006) this is due to the neutralisation of the amine groups on chitosan and the instability of particles close to the pKa of chitosan. Molecular weight had an impact on zeta potential values, but to a lesser extent than pH. It can be seen in Table 1 that LMWC in water and in an acidic solution had greater surface charges than their MMWC counterparts, which is consistent with work by Gan *et al*. (2005) who found that as molecular weight decreased, zeta potential increased.

Table 2

Chitosan nanosuspension pH	
Low Molecular Weight Chitosan + Water	6.82
Low Molecular Weight Chitosan + 1% Acetic acid	2.92
Medium Molecular Weight Chitosan + Water	6.89
Medium Molecular Weight Chitosan + 1% Acetic acid	2.99

When observing the nanoparticle solutions under the AFM (Figure 1), the particles displayed an irregular shape. Chitosan nanoparticles in acetic acid, as shown in Fig. 1 (A and C) exhibited uneven, but distinct outlines, whereas the nanoparticled chitosan in water solutions Fig. 1 (B and D) were less individualised and had jagged, blurred boundaries. The morphological characteristics of the chitosan nanoparticles in an acidic solution were similar to images obtained by Bugnicourt *et al*. (2014), and interestingly, chitosan nanoparticles in water resembled the appearance of bulk chitosan (Antoniou *et al*., 2015).

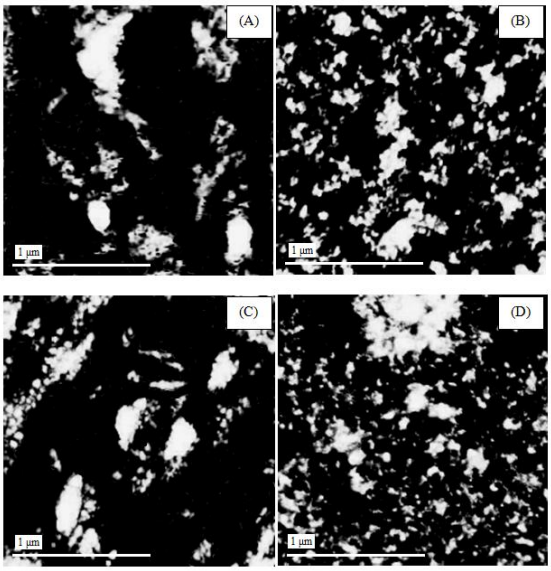


Figure 1 - Atomic Force Micrographs of: (A) LMWC + Acetic acid, (B) LMWC + Water, (C) MMWC + Acetic acid, and (D) MMWC + Water.

6.3.2 Antimicrobial Assessment of Manufactured Chitosan Nanoparticles

The MIC (%, w/v) of 0.25% chitosan nanoparticles dissolved in both ultrapure water and in 1% acetic acid solution against various Gram-negative, Gram-positive, Emmental and cottage cheese cultures are displayed in Table 3. LMWC and MMWC nanoparticles, when dissolved in ultrapure water, achieved the same inhibition (MIC = 0.250%) for all cultures examined. This result is similar to work conducted by Qi *et al*. (2004) who determined that chitosan nanoparticles in water carry an antimicrobial influence, but that this effect was not as strong as nanoparticles dissolved in acetic acid. These chitosan nanoparticles have some advantages over their bulk counterparts, namely; the nanoparticles are soluble in water alone and possess a positive charge (Table 1), even at neutral pH values (Table 2). These characteristics are most likely due to ionic forces exerted by TPP on chitosan (Käuper and Forrest, 2006). TPP possesses several pKa values, ranging from 1 to 8.5 (Lim and Seib, 1993), which may increase the pKa of chitosan-TPP nanoparticles as

compared to the pKa of chitosan alone (6.2-6.5). Despite attaining solubility in water, these forces do not seem to be sufficient to sustain stability in the fresh preparations in neutral media as indicated by the reduced zeta potential and labile behaviour observed in the AF micrographs and the somewhat mediocre inhibition levels. A pH region of 4-6 is recommended to ensure stability within chitosan nanoparticle dispersions (Nasti *et al*., 2009), whereas these nanoparticles are within the pH range of 6-7 (Table 2).

Table 3

Minimum inhibition concentration (MIC - % ,w/v), standard deviation and significance of nanoparticles against tested cultures

	LMWC + Water	LMWC + 1% Acetic acid	MMWC + Water	MMWC + 1% Acetic acid
Cottage Cheese	0.250 ± 0.00^{b}	0.083 ± 0.00^{a}	0.250 ± 0.00^{b}	0.083 ± 0.00^{a}
Emmental	0.250 ± 0.00^{b}	0.028 ± 0.00^{a}	0.250 ± 0.00^{b}	0.028 ± 0.00^{a}
Escherichia coli	0.250 ± 0.00^{b}	0.028 ± 0.00^{a}	0.250 ± 0.00^{b}	0.028 ± 0.00^{a}
Pseudomonas fluorescens	0.250 ± 0.00^{c}	0.022 ± 0.01^{a}	0.250 ± 0.00^{c}	0.028 ± 0.00^{b}
Staphylococcus aureus	0.250 ± 0.00^{b}	0.083 ± 0.00^{a}	0.250 ± 0.00^{b}	0.083 ± 0.00^{a}
Bacillus cereus	0.250 ± 0.00^{b}	0.028 ± 0.00^{a}	0.250 ± 0.00^{b}	0.028 ± 0.00^{a}
Total	0.250 ± 0.00^{b}	0.045 ± 0.03^{a}	0.250 ± 0.00^{b}	0.046 ± 0.03^{a}

Values correspond to the mean MIC for six data points, ± denotes the standard deviation.

^{a,b,c} Different lower case superscript letters in the rows indicate significant differences between treatments ($P \leq 0.05$).

LMWC - Low Molecular Weight Chitosan, MMWC - Medium Molecular Weight Chitosan

The antimicrobial activity of chitosan nanoparticles (LMWC and MMWC) in 1% acetic acid solution were found to be significantly higher ($P \leq 0.05$) than that of chitosan nanoparticles in water, against all cultures assessed. In previous work (Chapter 4), using bulk chitosan at 0.25% in acetic acid showed similar findings, although the nanoparticles synthesised in this study demonstrated better antimicrobial activity than the corresponding bulk chitosan against Emmental, Gram-positive and Gram-negative species. However, within this nano range, there was no trend deciphered in terms of size affecting antimicrobial effectiveness. This was also confirmed by Du *et al*. (2009), who found order of nanometre size contributed little to antimicrobial effect. LMWC (0.022%) worked better for *Pseudomonas fluorescens* than MMWC (0.028%), which was determined to be significant ($P \leq$

0.05), but in general, it was found that molecular weight had only a minor impact on the performance of chitosan nanoparticles (in acetic acid, 1%), which is in agreement with Vila *et al*. (2004). Cottage cheese-derived cultures appeared to be less susceptible than Emmental cheese-derived cultures to the action of nanoparticles within an acidic medium. The pH of cottage cheese is relatively high (5 – 5.3) (Johnson, 2001), and this, in addition to the increased moisture content, can provide an environment conducive to greater microbial growth. It was also observed that chitosan nanoparticles in acetic acid exhibited greater antimicrobial activity against Gram-negative bacteria than Gram-positive bacteria. This phenomenon has been observed before by Du *et al*. (2009), and these individuals concluded that the increased activity was associated with the different characteristics of cell surfaces between Gram-negative and Gram-positive species.

Nanoparticles are theorised to have greater functionality because of the quantum size effect that occurs when particles are reduced in size to the nanometre. Due to the disruption of the secondary structure caused by the process of ionic gelation, smaller-sized chitosan possess an increased surface area and a greater cationic charge (Qi *et al*., 2004), which allows for an increased affinity for interaction and increased likelihood of penetration potential. The most accepted mechanism proposed for the action of chitosan nanoparticles is the strong complexation that occurs between positively charged chitosan nanoparticles and the negative charge on the cell surface of microbial cells (Käuper and Forrest, 2006). This interaction results in an alteration of cell structure, particularly disrupting membrane integrity, thereby affecting permeability and allowing the release of intracellular contents, inducing bacterial death (Chung *et al*., 2004). It is clear from the above findings that when dispersed in an acidic medium, nanoparticles are particularly effective at achieving

microbial reduction. Dispersing medium, and therefore pH and zeta potential as a result, appear to contribute to the improvement of stability and antimicrobial performance. The addition of acetic acid elicits a decrease in pH and an increase in positive zeta potential due to the enhanced protonation of chitosan nanoparticles (Fan *et al*., 2012). The increased positive charge favours a greater degree of interaction with the negatively charged microbial cell surface to exhibit higher inhibitory activities (Shi *et al*., 2006).

Furthermore, chitosan nanoparticles in water attained moderate levels of inhibition; and it was previously shown that 1% acetic acid also contributed to antimicrobial activity (Chapter 4). However, neither acetic acid nor chitosan when administered individtually, could accomplish the same inhibitive qualities of chitosan and acetic acid in combination. It is clear that the unification of chitosan and acetic acid together exhibits a synergistic relationship of action. Future work should focus on this synergistic relationship, particularly in nanoparticle form, and incorporating these nanoparticles into films. The results demonstrated in this study, suggest that further investigation into the use of chitosan nanoparticles (in an acidic medium) and food applications, specifically cheese products, is warranted.

6.4 CONCLUSION

Small-sized particles with a modest size distribution and positive zeta potential were produced. The nanoparticles synthesised, although irregular in shape, were successfully soluble in both ultrapure water and 1% acetic acid. Nanoparticles in acetic acid demonstrated a more stable behaviour, and an increased antimicrobial activity, most likely due to their high positive surface charge. Nanoparticles (1% acetic acid) displayed an inhibitive effect against all cultures including cheese, and were more effective at reducing Gram-negative species than Gram-positive species. Comparing the antimicrobial properties of nanoparticles in 1% acetic acid to nanoparticles in water, it is clear that the antimicrobial effect between chitosan and acetic acid is a synergistic relationship. Nanoparticles in an acidic medium show promise as antimicrobial agents in the area of food packaging, particularly for use with cheese products. Future work should focus on developing a packaging incorporating the nanoparticles and observing their effect on application.

CHAPTER 7

Efficacy of antimicrobial HPMC-based films containing nanoparticled rosemary extract and chitosan (bulk and nanoparticled) *in vitro* and during storage of cheddar cheese at 4 °C and 12°C

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ABSTRACT

The objective of this study was to manufacture HPMC-based films containing bulk chitosan, laboratory prepared chitosan nanoparticles, and commercially-sourced nanoparticled rosemary extract at various concentrations. The optimum concentration for activity of each antimicrobial film was determined by liquid media inhibition. The films which demonstrated the greatest activity for each antimicrobial were further assessed by evaluating their antimicrobial efficacy when applied to *E. coli*-inoculated cheddar cheese stored at 4 °C and 12 °C. Antimicrobial solutions were successfully incorporated into the HPMC-based films and retained their inhibitory effect against the growth of *E. coli in vitro*. Nanoparticled rosemary extract and nanoparticled chitosan were most effective at controlling *E. coli* and yeast and mould growth in cheese, respectively, even at elevated temperatures. Results indicated that smaller particle sizes produced a greater antimicrobial effect, and in the case of nanoparticled rosemary extract, its function is enhanced upon *in vivo* application. Antimicrobial HPMC-based films demonstrate considerable potential for use on cheese products.

7.1 INTRODUCTION

As a result of increased global trade, the evolution of packaging science and higher product expectations in terms of quality and safety from consumers and industry, greater demands have been placed on the performance of everyday packaging. Consequently, certain technological fields, particularly in the area of smart packaging have been thrust to the forefront of research. Specifically, antimicrobial packaging has been highlighted in the area of safety and quality of foods. Antimicrobial packaging functions to delay, reduce, or inhibit the growth of microorganisms that are present within the packaged product by incorporating, coating or immobilizing antimicrobial agents to the polymer, addition of antimicrobial sachets or pads, or by using innately antimicrobial polymers (Appendini and Hotchkiss, 2002). In particular, the area of additive substances derived from natural resources is of definite interest due to their prospective interaction with food and their low- or no-risk upon ingestion. Chitosan is a derivative of chitin (a natural polysaccharide sourced predominately from crab and shrimp shells), which is widely used due to its cationic character, non-toxicity, biodegradability, biocompatibility, and its efficiency against bacteria, viruses and fungi (Rinaudo *et al*., 2006). Chitosan has been studied in many applications, as an antimicrobial agent, but mainly as an inherent antimicrobial polymer, usually in combination with other antimicrobials or polymers, against various food products. Emmental (Coma *et al*., 2002), Mozzarella (Duan *et al*., 2007), Saloio (Fajardo *et al*., 2010), Ricotta (Di Pierro *et al*., 2011), Cheddar (Moreira *et al*., 2011), are a few of the undertakings exploring the area of chitosan and cheese.

However, in recent times, nanotechnology is progressively recruited within the domain of antimicrobial packaging. Reducing the size of particles like antimicrobial

agents to a nanoscale range result in an increase in surface area to volume ratio, thereby leading to an improvement in reactivity (Neethirajan and Jayas, 2011), which can potentially enhance and expand antimicrobial performance. Nanoparticled chitosan synthesized by ionic gelation has been the focus of a number of studies, but to a much lesser extent than its bulk counterpart, despite the cytotoxicity of chitosan nanoparticles being no different to bulk chitosan (Huang *et al*., 2004). This antimicrobial has been incorporated into films (De Moura *et al*., 2009; Chang *et al*., 2010; Hosseini *et al*., 2015), with only Antoniou *et al*. (2015) examining its antimicrobial effect within a film during *in vitro* studies. Although nanoparticled chitosan films have been implicated for application in food packaging, it has gathered minimal attention with regard to actual food product applications. Ramezani *et al*. (2015) assessed the antimicrobial action of nanochitosan solutions dip-coated onto silver carp fillets, but from extensive review of the scientific literature, no studies have determined their application to cheese. Another natural nano-additive with potential for use in food contact materials is nanoparticled rosemary extract, which contains carnosoic acid at the core of its micellular structure. Rosemary is principally known for producing an antioxidant effect, but it has also demonstrated antimicrobial activity, with carnosoic acid being one of the main compounds present in rosemary responsible for its antimicrobial action (Moreno *et al*., 2006). However, nanoparticled rosemary has received little investigation. Vaka *et al*. (2013) evaluated a carnosic acid nanoparticulate system for upregulation of neurotrophins in the brain, but no other research has examined its use as an active agent within a film for food application.

Hydroxypropyl methylcellulose (HPMC) is a modified relative of cellulose. HPMC is odourless, tasteless and makes a suitable carrier film as it has been approved for

food applications (Burdock, 2007). It has been used in a number of antimicrobial assessment studies, which mainly focussed on its use with nisin as an active agent (Coma *et al*., 2001; Guiga *et al*. 2009; Imhran *et al*., 2010). Hard cheese, in particular, is an appropriate vehicle for assessing HPMC-based film activity because; spoilage occurs at the product surface, it has flat smooth surfaces with all sides capable of being in direct contact with the film, it is a perishable commodity which requires refrigeration and it is greatly affected when subject to temperature abuse. Therefore, the purpose of this study was to manufacture chitosan nanoparticles, prepare HPMC-based films containing these manufactured nanoparticled chitosan, commercially-sourced nanoparticled rosemary extract and bulk chitosan, and determine the optimum activity of these films *in vitro* against *E. coli* using liquid media inhibition. The efficacy of the films on application was also assessed by performing storage trials at 4 °C and 12 °C on *E. coli*-inoculated cheddar cheese.

7.2 MATERIALS AND METHODS

7.2.1 Materials

Chitosan nanoparticles were produced using ultrapure water (PURELAB Option-Q, Elga, UK) and medium molecular weight chitosan (190-310 kDa), glacial acetic acid, sodium hydroxide (NaOH), and sodium tripilyphosphate (TPP), all sourced from Sigma-Aldrich (St. Louis, MO, USA). Nanoparticled rosemary extract (carnosic acid) is a water- and fat-soluble solubilisate, which is pH, temperature and mechanically stable (Aquanova AG, Darmstadt, Germany). Low molecular weight chitosan (50-190 kDa) and hydroxypropyl methyl cellulose (HPMC) were both acquired from Sigma-Aldrich also. All antimicrobial solutions and films were prepared using distilled water. Tryptone Soya Agar (TSA), Mueller-Hinton Broth (MHB) and peptone water were purchased from Oxoid Ltd. (Basingstoke, Hampshire, UK), and Milk Plate Count Agar (MPCA), Tryptone Bile X-GLUC (TBX) Agar and Compact Dry Yeast and Mould (YM) were obtained from Lab M Limited (Lancashire, UK), Biolife (Milan, Italy) and Nissui Pharmaceuticals Co. Ltd. (Tokyo, Japan – supplied by Hyserve GmbH & Co., Uffing, Germany), respectively. Cheddar cheese was sourced locally.

7.2.2 Chitosan nanoparticle manufacture

Chitosan nanoparticles are the result of an ionic interaction between the positively charged primary amino groups of chitosan and the negatively charged phosphate groups of TPP (Fan *et al*., 2012). Chitosan nanoparticles were manufactured as described in Chapter 6. Briefly, chitosan was prepared at 0.3% (w/v) in 1% (v/v) acetic acid aqueous solution, stirred overnight and the pH adjusted to 4.6 using 10 N

NaOH. The cross-linking of chitosan with TPP (0.3% w/v) at a ratio of 5:1 was performed through stirring, by adding TPP at room temperature. The solution was stirred for a further 20 min, sonicated (80% amplitude) and centrifuged at 12000 *g* for 15 min. The supernatant was discarded and the precipitate was resuspended in water, followed by repetition of the sonication and centrifugation steps. The nanoparticles were then collected and prepared as antimicrobial solutions.

7.2.3 Antimicrobial solution preparation and characterisation

Antimicrobials and their concentration levels were chosen based on previous chapter work (Chapters 4, 5, and 6). Nanoparticled rosemary extract (NP-ROSE) was prepared at 0.5%, 1.0% 1.5% (v/v) in distilled water. The extract and water were heated to 40°C prior to mixing. Bulk chitosan (B-LMWC) and nanoparticled chitosan (NP-MMWC) were dissolved at 0.083%, 0.166% and 0.249% (w/v) in 1% (v/v) acetic acid solution. The antimicrobial solutions which demonstrated the greatest inhibition were further characterized. Particle size (d.nm), polydispersity index (PDI) and zeta potential measurements were performed at 173° backscatter angle at 25 °C using a Zetasizer Nano ZS (Malvern, Worcestershire, UK). Each dispersion was measured in triplicate and reported as the mean ± standard deviation.

7.2.4 HPMC film formation

Several potential materials including carrageenan, carboxymethylcellulose, gum arabic, HPMC, sodium casinate and starch, were all assessed as potential active carriers. HPMC was selected due to its compatibility with the antimicrobials to be

evaluated. The control film (Figure 1) was manufactured by dissolving HPMC (3%, w/v) in distilled water. This dissolution was achieved by heating a 1/3 of the volume of distilled water at 90 °C in a water bath under agitation (SW23, Julabo USA Inc., PA, USA). The HPMC powder was then added and allowed to dissolve. The slurry was removed from the water bath and the remaining two thirds of distilled water, at room temperature, was immediately added. The solution was then gently and magnetically stirred for 45 minutes and pH recorded. The film forming solution was cast into level Petri dishes (14 cm diameter) in aliquots of ~18 ml. The dishes were subsequently stored in an environmental climatic chamber for 48 hr at 25 °C and 50.5% relative humidity.

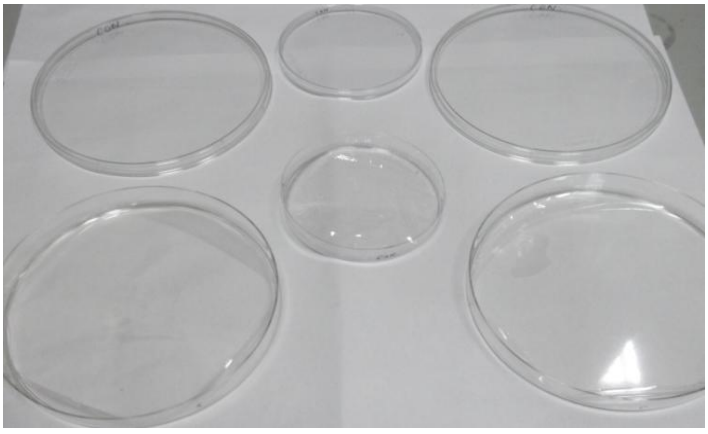


Figure 1 – Control HPMC film

Two methods of antimicrobial application were investigated - incorporation during film manufacture, and, spray coating post-film manufacture. However, spray treatment of the antimicrobial solutions was ultimately not employed due to inconsistent antimicrobial dispersion on the film surface. Therefore, antimicrobials

were incorporated into HPMC-based films under the same procedure as the control film manufacture above, except in this situation the distilled water portion was replaced with an antimicrobial solution (Figure 2). Ten films were created overall; Control, 0.5% NP-ROSE, 1.0% NP-ROSE, 1.5% NP-ROSE, 0.083% NP-MMWC, 0.166% NP-MMWC, 0.249% NP-MMWC, 0.083% B-LMWC, 0.166% B-LMWC, and 0.249% B-LMWC. All manufactured HPMC films, including those containing antimicrobials, were transparent and flexible. NP-ROSE films possessed a slight odour coupled with a visual observation of a yellowish, brown hue. Film thickness was calculated to the nearest 0.001 mm using a digital micrometer (Käfer Messuhrenfabrik GmbH & Co, Villingen-Schwenningen, Germany). Five measurements were recorded – one reading taken from the film centre and four readings taken from the film perimeter, which were used to determine the mean thickness. Control films had an average thickness of 29 µm, whilst HPMC films containing antimicrobials were slightly larger (NP-MMWC – 35 µm, B-LMWC – 39 µm, NP-ROSE – 50 µm).

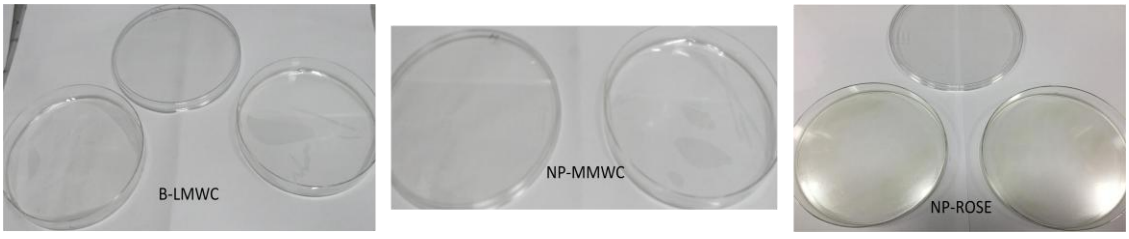


Figure 2 – Antimicrobial films (L to R: Bulk low molecular chitosan HPMC film, Nanoparticled medium molecular weight chitosan HPMC film, Nanoparticled rosemary HPMC film)

7.2.5 Culture preparation

The strain used for *in vitro* and in packaging application testing was Gram-negative species *Escherichia coli* (NCIMB 11943). *E. coli* was chosen as the examination strain for a number of reasons. Gram-negative species are generally less susceptible to the action of antimicrobial agents (Salton, 1994), with bulk chitosan (No *et al*., 2002), chitosan nanoparticles (Antoniou *et al*., 2015) and rosemary (Klančnik *et al*., 2009) all exhibiting a greater inhibitive effect on Gram-positive than Gram-negative bacteria. Additionally, *E. coli* is also a major microbiological concern for all cheese types (Hasell and Salter, 2003). Pasteurisation is employed to control its presence, but contamination can occur post-pasteurisation, and therefore it must be tested for on all commercial batches of cheese produced. Prior to testing, *E. coli* was maintained on a TSA slant at 4 °C. When it was required for use, a loopful of the strain was inoculated into 10 ml of sterile growth media (MHB) and incubated at 37 °C for 18 hr. The concentration of the suspension was determined to be 1.5 – 2.9 x 10^9 CFU/ml.

7.2.6 Antimicrobial activity of HPMC films – *in vitro* assay

The films were tested for their inhibition against *E. coli* using liquid media inhibition. Film samples were cut into squares (1 cm^2) and placed into individual test-tubes. A test-tube containing no film was also used. MHB (10 mls) was added to the tubes and immediately inoculated with 10 µl of the *E. coli* inoculum. The tubes were then incubated at 30 °C for 20 hr employing orbital shaking (120 rpm). Serial-dilutions of the resultant suspensions were made in peptone water and plated onto

TSA. Colonies were counted after incubation at 37 °C for 48 hr and reported as log CFU/ ml. Determinations were carried out in triplicate.

7.2.7 Antimicrobial efficacy of HPMC films on *E. coli*-inoculated cheddar cheese at 4 °C and 12 °C

In order to evaluate the antimicrobial effectiveness of films *in vivo*, the optimal performing concentration of each antimicrobial film from the *in vitro* assay (0.5% NP-ROSE, 0.166% NP-MMWC, 0.166% B-LMWC) was applied to cheddar cheese inoculated with *E. coli*. A no film treatment and a control HPMC film containing no antimicrobial were also tested, giving a total of five treatments.

The cheese was cut into rectangular cuboids (approximately 4 x 3 x 1 cm) weighing ~20 g each. *E. coli* was serially-diluted in peptone water (10^6 CFU/ml) and applied at several points to all sides of the cheese surface to achieve a final concentration of 10^4 CFU/g. Films were tightly wrapped around the inoculated cheese cuboid, whereby each side was in direct contact with the film. A no film control was prepared without wrapping. The samples, with and without film treatments, were then inserted within polyamide/polyethylene bags (Kompernass Handels GmbH, Bochum, Germany) and vacuum packaged. Day 0 measurement was determined immediately following inoculation. Microbial examinations occurred periodically over 28 and 14 days for 4 °C and 12 °C incubations, respectively, with assessments performed in triplicate. Cheddar cheese samples were examined for counts of total viable bacteria (medium - MPCA), *E. coli* (medium - TBX), and yeasts and moulds (medium - Compact Dry YM). For measurement, cheese was aseptically transferred to a sterile stomacher bag and homogenized with 180 ml peptone water for 1 min. Ten-fold serial dilutions

were carried out and appropriate aliquots of the resultant suspensions (0.1 ml – MPCA and TBX, 1 ml – Compact Dry YM) were plated onto their corresponding media. *E. coli* plates were incubated at 37 °C for 24 hr, and total viable count and yeasts and moulds were incubated at 30 °C for 3 days and 5 days, respectively. Colonies were counted and expressed as log CFU/g.

7.2.8 Statistical analysis

The *in vivo* experimental data was analysed on SPSS Statistics 20 (IBM, Armonk, NY, USA). One-way ANOVA and Tukey's Post-Hoc tests were applied to determine statistical significance between treatments and differences over time within a treatment. The level of significance was set at $P \leq 0.05$ - 0.01 (significant), $P \leq 0.01$ - 0.001 (highly significant), $P \leq 0.001$ (extremely significant). Means with the letters 'ns' are non-significant, $P > 0.05$.

7.3 RESULTS AND DISCUSSION

7.3.1 Antimicrobial activity of HPMC films - *in vitro*

The antimicrobial activity of HPMC films evaluated against Gram-negative *E. coli* via liquid media inhibition is shown (Figure 3). The treatment containing no film had the highest log CFU/ml value (9.1). The control HPMC film, without any antimicrobial present, demonstrated some inhibition (8.9 log CFU/ml, which is a reduction compared to the no film treatment). Previous investigations examining the inhibitive effect of stand-alone HPMC films have displayed no antimicrobial properties, including tests against *E. coli* (Chana-Thaworn *et al*., 2011; Sánchez-González *et al*., 2011).

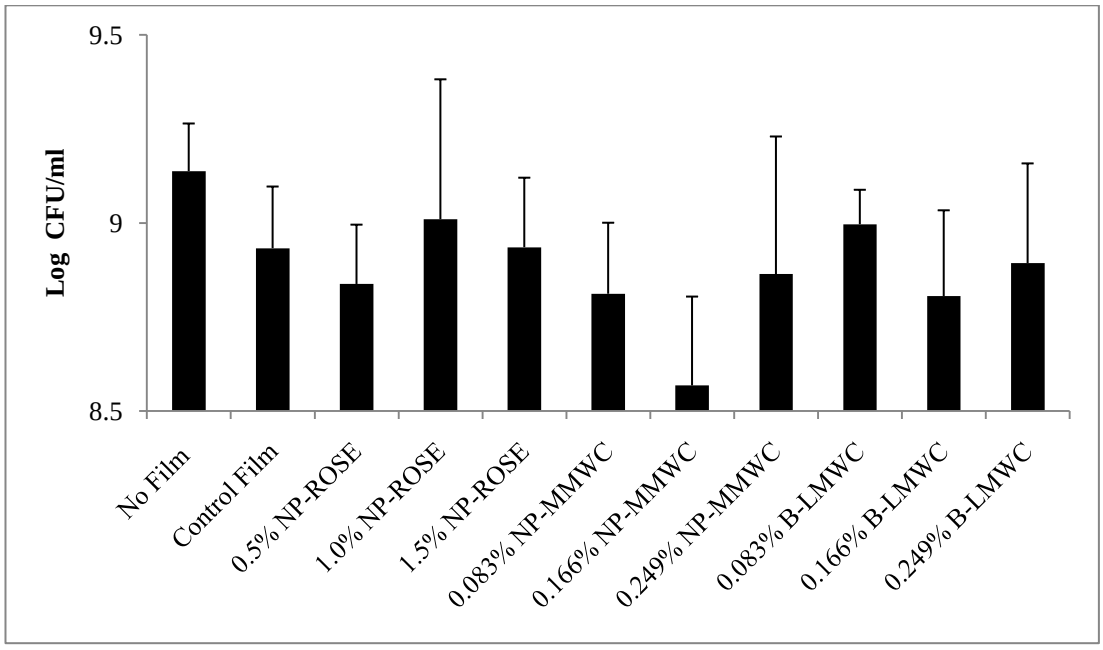


Figure 3 - Antimicrobial activity of HPMC film treatments *in vitro* against *E. coli*. Treatment effect expressed as log CFU/ml. Error bars represent standard deviation.

However, it was the presence of 0.166% NP-MMWC, 0.166% B-LMWC, 0.083% NP-MMWC, 0.5% NP-ROSE and 0.249% NP-MMWC, which provided the greatest inhibitive activity of 8.6, 8.8, 8.8, 8.8, and 8.9 log CFU/ml, respectively. It appears

that all concentrations of NP-MMWC exhibited some antimicrobial activity, although no trend is discernable in terms of concentration increasing or decreasing. This finding differs from Huang *et al*. (2009), who found that antimicrobial activity increased when chitosan nanoparticle concentration increased. The size of nanoparticles at each concentration, prior to insertion into the films, was measured using a Zetasizer (Table 1). Again, no noticeable pattern between level of activity and nanoparticle size was recognised, an observation which was also confirmed by Du *et al*. (2009). From the results of this assay, the HPMC films with the best performing concentration of each antimicrobial (0.5% NP-ROSE, 0.166% NP-MMWC, 0.166% B-LMWC) were selected for examination against *E. coli* inoculated cheddar cheese.

Table 1

Size and polydispersity index (PDI) of NP-MMWC

	Particle Size (nm)	PDI
0.083% NP-MMWC	146.4 ± 18.59	0.477 ± 0.03
0.166% NP-MMWC	216.5 ± 05.69	0.287 ± 0.01
0.249% NP-MMWC	189.3 ± 16.06	0.517 ± 0.06

NP-MMWC - Nanoparticled Medium Molecular Weight Chitosan

7.3.2 Antimicrobial activity of HPMC films applied to cheese at 4 °C and 12 °C

The efficacy of various treatments on *E. coli* inoculated cheddar cheese at 4 °C and 12 °C (a and b – Total viable count, c and d – *E. coli* count, e and f – Yeast and mould count) is shown (Table 2).

Table 2

Antimicrobial activity of HPMC film treatments on cheese stored at 4 °C and 12 °C. Data is expressed as log CFU/g, ± standard deviation.

(a) 4 °C - Total Viable Count

Treatment	Day 0	Day 1	Day 4	Day 7	Day 14	Day 21	Day 28	*P*
No Film	3.70 ± 0.02	3.57 ± 0.48	3.23 ± 0.07	3.55 ± 0.12^{c}	3.67 ± 0.14	3.86 ± 0.19^{ab}	4.53 ± 0.30	*
Control Film	3.70 ± 0.02	3.56 ± 0.27	3.40 ± 0.40	3.43 ± 0.00^{bc}	2.75 ± 0.21	3.99 ± 0.12^{ab}	4.71 ± 0.20	***
0.5% NP-ROSE	3.70 ± 0.02	3.20 ± 0.08	3.16 ± 0.11	2.95 ± 0.07^{a}	3.32 ± 0.09	3.54 ± 0.11^{a}	3.87 ± 0.55	*
0.166% NP-MMWC	3.70 ± 0.02	3.25 ± 0.49	3.24 ± 0.41	3.16 ± 0.02^{ab}	3.59 ± 0.36	3.82 ± 0.31^{ab}	4.06 ± 0.03	ns
0.166% B-LMWC	3.70 ± 0.02	3.40 ± 0.51	3.44 ± 0.19	3.36 ± 0.08^{bc}	3.38 ± 0.42	4.41 ± 0.02^{b}	5.09 ± 0.27	**

(b) 12 °C - Total Viable Count

Treatment	Day 0	Day 1	Day 4	Day 7	Day 14	*P*
No Film	3.70 ± 0.02	3.75 ± 0.07^{c}	3.48 ± 0.07	4.52 ± 0.12^{c}	5.48 ± 0.00	***
Control Film	3.70 ± 0.02	3.19 ± 0.10^{a}	3.48 ± 0.06	4.25 ± 0.05^{bc}	5.48 ± 0.00	***
0.5% NP-ROSE	3.70 ± 0.02	3.16 ± 0.06^{a}	3.05 ± 0.21	3.99 ± 0.02^{ab}	5.31 ± 0.25	***
0.166% NP-MMWC	3.70 ± 0.02	3.44 ± 0.09^{ab}	3.16 ± 0.54	3.75 ± 0.02^{a}	4.69 ± 0.41	*
0.166% B-LMWC	3.70 ± 0.02	3.64 ± 0.03^{bc}	3.65 ± 0.06	4.15 ± 0.10^{b}	4.95 ± 0.12	***

(c) 4 °C - *E. coli* Count

Treatment	Day 0	Day 1	Day 4	Day 7	Day 14	Day 21	Day 28	*P*
No Film	2.93 ± 0.46	3.08 ± 0.05	3.11 ± 0.21	3.09 ± 0.27	3.24 ± 0.23	3.24 ± 0.23	3.31 ± 0.15^{b}	ns
Control Film	2.93 ± 0.46	2.96 ± 0.26	3.28 ± 0.00	2.69 ± 0.13	2.35 ± 0.49	2.39 ± 0.13	2.65 ± 0.07^{a}	ns
0.5% NP-ROSE	2.93 ± 0.46	2.80 ± 0.14	2.78 ± 0.25	1.30 ± 1.84	1.35 ± 1.91	1.00 ± 1.41	2.15 ± 0.21^{a}	ns
0.166% NP-MMWC	2.93 ± 0.46	3.16 ± 0.29	2.94 ± 0.34	2.95 ± 0.24	2.94 ± 0.34	2.85 ± 0.00	2.63 ± 0.21^{a}	ns
0.166% B-LMWC	2.93 ± 0.46	2.99 ± 0.13	2.85 ± 0.21	2.66 ± 0.26	2.77 ± 0.10	2.73 ± 0.17	2.54 ± 0.09^{a}	ns

(d) 12 °C - *E. coli* Count

Treatment	Day 0	Day 1	Day 4	Day 7	Day 14	*P*
No Film	2.93 ± 0.46	3.34 ± 0.48	3.20 ± 0.23	3.03 ± 0.25	2.77 ± 0.10^{b}	ns
Control Film	2.93 ± 0.46	2.90 ± 0.08	2.48 ± 0.68	2.72 ± 0.17	2.87 ± 0.04^{b}	ns
0.5% NP-ROSE	2.93 ± 0.46	3.06 ± 0.03	2.60 ± 0.00	2.60 ± 0.43	0.00 ± 0.00^{a}	***
0.166% NP-MMWC	2.93 ± 0.46	3.16 ± 0.29	2.39 ± 0.13	2.42 ± 0.60	2.60 ± 0.00^{b}	ns
0.166% B-LMWC	2.93 ± 0.46	3.18 ± 0.39	3.03 ± 0.25	2.87 ± 0.24	2.30 ± 0.43^{b}	ns

(e) 4 °C - Yeast and Mould Count

Treatment	Day 0	Day 1	Day 4	Day 7	Day 14	Day 21	Day 28	*P*
No Film	0.65 ± 0.92	1.70 ± 0.00	2.85 ± 0.03^{b}	3.05 ± 0.09^{c}	4.10 ± 0.02^{c}	5.32 ± 0.04^{e}	5.50 ± 0.71^{a}	***
Control Film	0.65 ± 0.92	2.42 ± 0.11	2.78 ± 0.07^{b}	3.13 ± 0.03^{c}	3.15 ± 0.21^{b}	4.93 ± 0.03^{d}	6.93 ± 0.03^{b}	***
0.5% NP-ROSE	0.65 ± 0.92	1.00 ± 0.00	0.65 ± 0.92^{a}	1.24 ± 0.34^{ab}	3.24 ± 0.01^{b}	3.95 ± 0.00^{a}	5.02 ± 0.09^{a}	***
0.166% NP-MMWC	0.65 ± 0.92	0.50 ± 0.71	0.50 ± 0.71^{a}	0.65 ± 0.92^{a}	2.60 ± 0.05^{a}	4.44 ± 0.01^{b}	5.00 ± 0.00^{a}	***
0.166% B-LMWC	0.65 ± 0.92	0.65 ± 0.92	2.08 ± 0.05^{ab}	2.90 ± 0.01^{bc}	3.50 ± 0.01^{b}	4.70 ± 0.07^{c}	5.76 ± 0.04^{ab}	***

(f) 12 °C - Yeast and Mould Count

Treatment	Day 0	Day 1	Day 4	Day 7	Day 14	*P*
No Film	0.65 ± 0.92	3.04 ± 0.08^{b}	3.07 ± 0.05^{c}	3.48 ± 0.00^{b}	4.48 ± 0.00	***
Control Film	0.65 ± 0.92	1.95 ± 0.07^{ab}	3.48 ± 0.00^{d}	3.48 ± 0.00^{b}	4.48 ± 0.00	***
0.5% NP-ROSE	0.65 ± 0.92	0.65 ± 0.92^{a}	1.59 ± 0.16^{a}	3.48 ± 0.02^{b}	4.48 ± 0.00	***
0.166% NP-MMWC	0.65 ± 0.92	0.65 ± 0.92^{a}	1.69 ± 0.13^{a}	3.24 ± 0.01^{a}	4.48 ± 0.00	***
0.166% B-LMWC	0.65 ± 0.92	1.70 ± 0.00^{ab}	2.36 ± 0.08^{b}	3.48 ± 0.00^{b}	4.48 ± 0.00	***

NP-ROSE - Nanoparticled Rosemary Extract, NP-MMWC - Nanoparticled Medium Molecular Weight Chitosan, B-LMWC - Bulk Low Molecular Weight Chitosan.

Letters within columns represent differences between treatments on each day. If no letter is present within a column this indicates no significance between treatments on that day.

P value represents significance over time within each treatment. The level of significance is denoted by these asterisks; * = $P \leq 0.05$ - 0.01 (significant), ** = $P \leq 0.01$ - 0.001 (highly significant), *** = $P \leq 0.001$ (extremely significant).

Means with the letters 'ns' are non-significant, $P > 0.05$.

- *Total viable count*

In general, counts at both storage temperatures (4 °C and 12 °C) experienced a slight decrease, followed by a slight increase, in growth over time and this was significant for all treatments, with the exception of 0.166% NP-MMWC. As anticipated, cheese samples stored at 12 °C deteriorated faster than those stored at 4 °C. By the final day of storage, NP-ROSE had the lowest log CFU/ml (3.87) at 4 °C, and NP-MMWC had the lowest log CFU/ml (4.69) at 12 °C, but neither of these effects was significant.

The viable microbial count is inclusive of those microorganisms associated with cheese ripening. Microbiological changes of starter bacteria and non-starter microflora contribute to the ripening process and are partly responsible, and therefore essential, in achieving the established characteristic flavour, aroma, texture and appearance of cheese (Singh *et al.*, 2003). It may be advantageous that the general microbial count of the film treatments is similar to the no film treatment, as it demonstrates that the antimicrobials do not greatly affect the overall complex cheese microflora. If the antimicrobial treatments exhibited an inhibitive effect on the desirable microorganisms, then the sensorial properties of the cheese may be affected. Consumers have previously vocalised that any alteration to product organoleptic quality is unacceptable, regardless of any additional benefits or functions imposed (Chapter 2). Therefore, it is imperative when developing antimicrobial packaging, that the antimicrobials must perform against unwanted microorganisms without interfering excessively with the native microbiota of the cheese. For that reason the action of these films against *E. coli* and yeasts and moulds is more important as they represent the true targets of these antimicrobials.

- *E. coli count*

The control film and the no film treatment experienced the highest levels of growth by the final day of storage (4 °C and 12 °C). B-LMWC and NP-MMWC were found to be the second and third most effective antimicrobial film treatments for both storage temperatures. However, it was NP-ROSE which demonstrated the greatest antimicrobial influence. At 4 °C, levels of *E. coli* with NP-ROSE films were the lowest on any day examined and this was significantly ($P \leq 0.05$) different from the no film treatment by day 28. NP-ROSE was particularly effective against *E. coli* at 12 °C, and on the final day of the trial it was significantly ($P \leq 0.05$) different from all other treatments. With the exception of the no film treatment at 4 °C, all treatments brought about a decrease in microbial numbers by the end of the trial compared to day 0. In general it was noted that the cheese samples held at 12 °C experienced a faster decrease in *E. coli* population compared to cheese samples stored at 4 °C on day 14 of storage. This is consistent with findings by Suppakul *et al*. (2008), who explained this phenomena by suggesting that the higher the temperature, the higher the release rate of the active agent from the film to the food surface and consequently, microbial reduction is achieved at a greater pace.

In contrast to our *in vitro* results, whereby 0.166% NP-MMWC, 0.166% B-LMWC, and 0.5% NP-ROSE were the most effective treatments against *E. coli* (in this order); the opposite was found upon application to the cheese. This may occur because *in vitro* assessments often fail to replicate the actual conditions of *in vivo* (Tunev, 2012). Additionally, further antimicrobial activity may be achieved, in this case for NP-ROSE, when the antimicrobial polymer is applied directly to a solid food surface (Perez-Perez *et al*., 2006), however, Huang *et al*. (2009) also alluded to the intrinsic properties of bulk chitosan, thereby restricting its use *in vivo*.

- *Yeast and mould count*

Yeast and mould counts for both 4 °C and 12 °C increased significantly ($P \leq 0.001$) over time, with samples stored at 12 °C displaying accelerated growth. At 4 °C on the final day of storage, NP-MMWC and NP-ROSE produced the lowest log CFU/ml values, 5.00 and 5.02, respectively, which were significantly different ($P \leq 0.05$) from the control film treatment. It was observed that at 12 °C, NP-MMWC provided the longest delay in yeast and mould growth. On day 4 of storage, both NP-ROSE and NP-MMWC demonstrated an effect that was significantly lower than all other treatments, and on day 7, NP-MMWC continued to influence growth ($P \leq 0.05$), but by day 14, all treatments performed to the same degree. The early detection of fungal presence is important for cheese as they are often the first visible indication of spoilage to the consumer. Fungal spoilage often occurs in cheese due to its low pH, favourable nutritional profile, and the frequent presence of surface moisture, which can manifest itself in cheese by changes in colour, texture, off-odours and flavours (Ledenbach and Marshall, 2010). However, there was no visual colony observed on the cheese surface at either 4 °C or 12 °C at the end of the storage period.

7.3.3 Overall activity and relationship to size

Results from this work demonstrated that NP-ROSE and NP-MMWC were the most effective at inhibiting *E. coli* and yeasts and moulds, respectively, and performed better than the no film treatment for all microbiological assessments. The control HPMC failed to display any antimicrobial activity and for certain conditions, particularly at 12 °C, had a higher log CFU/ml than the treatment containing no film.

B-LMWC exhibited a moderate level of antimicrobial activity overall. The antimicrobial action of chitosan is due to its polycationic nature which can interact with the negatively charged components of the microbial surface, which subsequently affects the cell permeability and results in leakage of cell contents (Park and Kim, 2010). Despite bulk chitosan recording a greater positive charge than nanoparticled chitosan (Table 3), nanoparticled chitosan exhibited greater activity. Positively-charged chitosan crosslinks at a ratio of 5:1 with negatively-charged TPP, leading to a formation of chitosan nanoparticles. This interaction results in a reduced positive charge on the nanoparticles due to TPP binding with some of the positive sites on the chitosan surface. Therefore, the decreased size and increased surface area of the chitosan nanoparticles provides a higher affinity for complexation with the corresponding microorganism (Qi *et al*., 2004). However, the zeta potential of NP-ROSE measured a negative charge (Table 3), suggesting that the observed antimicrobial activity is unlikely to be an electrostatic interaction, or pH related (Table 3), and is more likely to be ascribed to its encapsulated nature and reduced particle size. Georgantelis *et al*. (2007) discussed how phenolic diterpenes, like carnosic acid, have a reduced ability to penetrate the cell membrane, particularly of Gram-negative bacteria. It is possible that this nanoparticled rosemary extract based on carnosic acid, due to being encapsulated within a surfactant is able to infiltrate the cell, including the outer membrane of Gram-negative species, as shown by the activity against *E. coli*. Prabhu and Poulose (2012) have previously alluded to the concept that a decreased particle size, specifically those in the nano-scale, have an increased penetration potential. Therefore, NP-ROSE measured the smallest diameter (63.59 ± 0.65 nm), followed by NP-MMWC (182.5 ± 7.93 nm), and lastly B-LMWC (1349 ± 150.20 nm) (Table 3), and these results suggest that antimicrobial

activity coincides with size range and their associated penetration capabilities. This study has demonstrated the potential ability of antimicrobial HPMC-based films containing NP-ROSE and NP-MMWC on future food applications, particularly for cheese products.

Table 3

Size, polydispersity index (PDI), and zeta potential of the antimicrobial solutions and the pH of the associated film forming solution (FFS)

	Control Film	0.5% NP-ROSE	0.166% NP-MMWC	0.166% B-LMWC
Size	-	63.59 ± 0.65	182.5 ± 7.93	1349 ± 150.20
PDI	-	0.506 ± 0.01	0.349 ± 0.03	0.633 ± 0.07
Zeta Potential	-	-28.2 ± 0.10	54.8 ± 2.03	68.2 ± 1.83
pH of FFS	5.51 ± 0.04	5.10 ± 0.18	3.21 ± 0.32	3.17 ± 0.24

NP-ROSE - Nanoparticled Rosemary Extract, NP-MMWC - Nanoparticled Medium Molecular Weight Chitosan, B-LMWC - Bulk Low Molecular Weight Chitosan.

7.4 CONCLUSION

Antimicrobial solutions were successfully incorporated into the HPMC-based films and retained their inhibitory effect against the growth of *E. coli in vitro*, and, on direct film application against *E. coli* inoculated cheddar cheese. Tests executed *in vitro* demonstrated that all film treatments provided a microbial reduction compared to the no film control. NP-MMWC (0.166%), B-LMWC (0.166%), and NP-ROSE (0.5%) films exhibited the greatest inhibitive effect of each antimicrobial, and were applied to cheddar cheese to determine their efficacy *in vivo*. When these levels of NP-ROSE and NP-MMWC were applied to cheese, they were most effective at controlling *E. coli* and yeast and mould growth, respectively, even at elevated temperatures. Results indicated that smaller particle sizes produced a greater antimicrobial effect, and in the case of NP-ROSE, its function is enhanced upon *in vivo* application. Antimicrobial HPMC films demonstrate considerable potential for use on cheese products and future work should focus on the effect of these antimicrobial films on sensory properties and determine if their spectrum of activity can be expanded to the control of pathogenic bacteria, as well as other food products.

CHAPTER 8 - Overall Discussion, Conclusion and Future Research

8.1 OVERALL DISCUSSION

As cheese consumption grows globally and greater expectations are placed on packaging function, the application of smart packaging to cheese products expands. However, the route to implementation of such smart packaging technologies is still very much in its infantile stage, as demonstrated by the limited number of commercial examples in use in the current market (Chapter 1). Therefore, research, like the work undertaken in this thesis, is critical to demonstrate that smart applications are necessary and commercially relevant.

Acceptance of new technologies by all supply chain participants is essential for widespread commercial success. Often this level of acceptance is dependent on the technology in question and the product to which the technology is applied. Thus, consumer acceptance of the smart packaging technologies that were involved in this thesis were explored, with a specific focus on use with cheese products (Chapter 2). Consumers were in favour of the use of technology to extend cheese shelf-life, with the majority of respondents being open to the employment of all three technologies (intelligent, active and nanotechnology). However, some consumers questioned the need of such technologies and certain product applications of this technology were seen to be more beneficial, such as employment on soft, expensive, grated or sliced formats, or for use on cheese destined for export. It was also shown that acceptance increased after the provision of information. Therefore education and communication are key to the successful employment of smart technologies to cheese packaging.

In order to demonstrate the need for such technologies, current commercial cheese packaging was assessed in an industrial setting (Chapter 3). Intelligent oxygen sensors were applied to the surface of cheese on a commercial cheese packaging line

prior to being packaged. Cheese was stored both onsite and offsite, to evaluate packaging and packaging process function and to determine the effect of distribution on packaging containment. Oxygen levels increased in both storage treatments, establishing that the current packaging system employed was not fulfilling its full capability and function. However, oxygen levels were nearly 3-times higher (onsite – 0.37% vs. offsite – 1.05%) for those cheese packages transported offsite, thereby indicating distribution can catalyse package failure. Ultimately, intelligent sensors demonstrated relevance of immediate implementation to industry and also displayed the urgent need for better packaging systems, potentially those of an active antimicrobial nature, particularly for use with products destined for export.

In order to develop an active antimicrobial film suitable for use on cheese products with the purpose of extending shelf-life, several potential active agents were screened (Chapter 4). These agents included organic acids (sorbic acid, benzoic acid, acetic acid), chitosans (low- and medium-molecular weighted), and commercial nanoparticled solubilisates (sorbic acid, benzoic acid, curcumin, ascorbic acid and rosemary extract), examined at various levels. Nanoparticled sorbic acid and benzoic acid displayed the same activity, but possessed enhanced solubility compared to bulk organic acids. Nanoparticled rosemary demonstrated significant activity ($P \leq 0.001$) against cheese-derived cultures and Gram-positive bacteria, whereas curcumin and ascorbic acid nanoparticle solubilisates failed to produce any inhibition at <1%. Chitosan of both molecular weights exhibited the widest spectrum of activity at the lowest minimum inhibition concentration (MIC) levels. Assessing acetic alone demonstrated that it contributes some activity to the action of chitosan, but its inhibition concentration is much higher, concluding that chitosan accounts for the majority of the inhibition attained.

Agents which presented the most positive properties such as antimicrobial action or solubility were further examined in combination to determine possible synergistic relationships that may occur in terms of inhibition (Chapter 5). The agents advanced to this study were chitosan (low- and medium-molecular weight) and nanoparticled commercial solubilisates (sorbic acid, benzoic acid and rosemary extract). Of the combinations evaluated, no synergism was determined. Nanoparticled sorbic and benzoic acid produced modest activity and their effect in general was enhanced when in combination with chitosan, but this force was additive and not synergistic as the MIC levels were higher than observed for chitosan alone. The most influential treatments were resolved to be nanoparticled rosemary extract and bulk chitosan of both molecular weights. Rosemary nanoparticles re-demonstrated its selective action, displaying no activity against Gram-negative spoilage bacteria. Chitosan also proved to be most effective, with low-molecular weight chitosan exhibiting a slightly better function. It can be derived from the two active antimicrobial agent screening studies (Chapters 4 and 5), that chitosan, individually or in combination, provided the greatest activity overall, yielding the lowest MIC levels and inducing the broadest range of inhibition.

Progressing from this knowledge, this bulk chitosan was transformed into nanoparticled chitosan via ionic gelation and examined for its antimicrobial activity against cheese-derived cultures, as well as various spoilage bacteria (Chapter 6). The particles manufactured were in the nanometre range, stable, and demonstrated solubility in water alone. Compared to Chapters 4 and 5, in which chitosan alone could not be evaluated due to its insolubility in water, here chitosan nanoparticles in water were tested for antimicrobial action, and it is clear its activity is significantly different ($P \leq 0.05$) to chitosan nanoparticles suspended in acetic acid (Table 1),

therefore the combination of chitosan and acetic acid is a synergistic relationship. Nanoparticles in acetic acid displayed an inhibitive effect against all cultures assessed. When comparing the effect of bulk chitosan from Chapter 4 to nanoparticled chitosan (Table 1), when both dissolved in acetic acid, it was observed that whilst bulk and nanoparticled low molecular weight chitosan were not significantly different from each other ($P > 0.05$); bulk and nanoparticled medium molecular weight chitosan exhibited significantly different activity ($P \leq 0.05$).

Table 1

Comparision of results from Chapters 4 and 6, presented as the mean Minimum Inhibition Concentration (MIC, % w/v)

Antimicrobial Treatment	*Cottage Cheese*	*Emmental*	*Escherichia coli*	*Pseudomonas fluorescens*	*Staphylococcus aureus*	*Bacillus cereus*	*Total*
0.25% LMWC + Water † [c]	0.250 ± 0.00	0.250 ± 0.00	0.250 ± 0.00	0.250 ± 0.00	0.250 ± 0.00	0.250 ± 0.00	0.250 ± 0.00
0.25% LMWC + 1% Acetic acid † [a]	0.083 ± 0.00	0.028 ± 0.00	0.028 ± 0.00	0.022 ± 0.01	0.083 ± 0.00	0.028 ± 0.00	0.045 ± 0.03
0.25% MMWC + Water † [c]	0.250 ± 0.00	0.250 ± 0.00	0.250 ± 0.00	0.250 ± 0.00	0.250 ± 0.00	0.250 ± 0.00	0.250 ± 0.00
0.25% MMWC + 1% Acetic acid † [a]	0.083 ± 0.00	0.028 ± 0.00	0.028 ± 0.00	0.028 ± 0.00	0.083 ± 0.00	0.028 ± 0.00	0.046 ± 0.03
0.25% Bulk LMWC + 1% Acetic acid ‡ [a]	0.028 ± 0.00	0.028 ± 0.00	0.050 ± 0.30	0.028 ± 0.00	0.083 ± 0.00	0.050 ± 0.03	0.046 ± 0.03
0.25% Bulk MMWC + 1% Acetic acid ‡ [b]	0.028 ± 0.03	0.072 ± 0.00	0.083 ± 0.00	0.039 ± 0.03	0.116 ± 0.08	0.061 ± 0.03	0.067 ± 0.04
1% Acetic acid ‡	0.111 ± 0.00	0.111 ± 0.00	0.111 ± 0.00	0.096 ± 0.03	0.466 ± 0.30	0.096 ± 0.03	0.174 ± 0.16

† - Laboratory manufactured chitosan nanoparticles from Chapter 6.

‡ - Bulk chitosan and acetic acid from Chapter 4.

[a,b,c] Different lower case superscript letters in the Antimicrobial Treatments column indicate significant differences between treatments ($p<0.05$). Multiple comparisions were made for all chitosan solutions using Tukey.

Analysing the results from Chapters 4, 5, and 6, it was determined that bulk low-molecular weight chitosan, laboratory-manufactured nanoparticled medium molecular weight chitosan and commercially-sourced nanoparticled rosemary extract administered the largest spectrum of function. These were therefore selected for incorporation into an active antimicrobial film for examination against *Escherichia coli in vitro* and on application to *E. coli*-inoculated cheese (Chapter 7). The levels at which these antimicrobials were applied was determined by the highest MIC values determined from Chapters 4, 5 and 6 (bulk low-molecular weight chitosan – 0.083%, nanoparticled rosemary extract – 0.500%, nanoparticled medium-molecular weight chitosan – 0.083%). Concentrations were doubled and tripled as often increased application levels are required when examining antimicrobial responses in foods, as fat, protein, water and salt can affect microbial resistance (Shelef, 1984).

Hydroxymethyl cellulose (HPMC) was chosen a carrier material for these antimicrobials after screening compatibility with other films. Antimicrobials were successfully incorporated into the films and the different levels were assayed *in vitro* by liquid media inhibition to determine the optimum concentration for application. Bulk low-molecular weight chitosan (0.166%), laboratory-manufactured nanoparticled medium-molecular weight chitosan (0.166%) and commercially-sourced, nanoparticled rosemary extract (0.5%) demonstrated the greatest activity of each antimicrobial film and these films were advanced to the application stage. Nanoparticled chitosan and rosemary were most efficacious at controlling fungal and *E. coli* growth on cheese, respectively. In application, nanoparticles proved to be better in terms of performance, indicating particles of a reduced size produce an enhanced exertion.

In summary, intelligent packaging was used to demonstrate the need for packaging improvement, and active packaging, inclusive of nanoparticled agents, was implemented to provide an antimicrobial function. This thesis demonstrated the necessity of smart packaging technologies and how they can be integrated successfully into current packaging formats to develop a superior packaging suitable for cheese products, which should receive low consumer resistance, provided the cheese application is warranted and relevant information is disseminated to the public. Outcomes illustrate these technologies can benefit the economy, as well as all supply chain participants, and are commercially relevant as they identify packaging problems and provide an active antimicrobial function, both of which generate an improvement in final product quality and shelf-life. These improvements can advance the cheese industry to have an export driven focus and encourage smart innovation to be pushed further within the entire packaging industry.

8.2 OVERALL CONCLUSION

Results illustrate the inadequacy of current packaging and the ability of smart packaging technologies to improve established packaging formats.

- The consumer acceptance of smart packaging technologies like intelligent, active and nanotechnology is promising, but it is very much dependent on the cheese application and on the information disseminated to the public.
- Current commercial cheese packaging utilised in industry is not sufficient, particularly when subject to distribution, as determined using intelligent oxygen sensor technology.
- This work also demonstrated that this technology is presently industrially relevant.
- Bulk chitosan and nanoparticled rosemary extract displayed the greatest antimicrobial activity against cheese-derived cultures and spoilage bacteria, however, when in combination, synergism was not observed between these agents.
- Stable, laboratory-manufactured chitosan nanoparticles were produced by ionic gelation and these particles also exhibited inhibitive properties when assessed against cheese-derived cultures and Gram-negative and Gram-positive microorganisms.
- Bulk low molecular weight chitosan, nanoparticled medium molecular weight chitosan and nanoparticled rosemary extract were successfully incorporated into individual HPMC-based films.
- Chitosan nanoparticle films obtained the best antimicrobial activity *in vitro* versus *E. coli*.

- On application to cheese, films containing nanoparticled rosemary and nanoparticled chitosan demonstrated efficacy against *E. coli* and fungal growth, respectively.

In conclusion, smart packaging technology within the cheese sector has been shown to be both necessary and consumer acceptable, provide a range of functionalities specifically applicable and beneficial to cheese products, proven successful in *in vitro* operations and validated in application to cheese products.

8.3 FUTURE RESEARCH

This thesis presents exploratory work investigating the scope of function of smart packaging technologies and cheese packaging systems. The use of these technologies was shown to yield a diversified range of benefits. However, due to the limited extent of smart packaging systems actually applied to cheese products commercially, there is opportunity to further research and investigate these technologies in application. Future research arising from the work presented in the thesis are summarised as follows;

- Endeavour to increase the level of acceptance of technological application amongst all supply chain stakeholders and other relevant bodies such as government and regulatory agencies.
- Reduce the visibility of intelligent sensor presence within packaging.
- Broaden the active agent range.
- Expand assessment to include screening against pathogenic bacteria.
- Fully investigate migration and safety concerns of the packaging systems.
- Employ sensory evaluation to the cheese product contained within the smart packaging application.
- Observe the effects of long term storage using the developed packaging system.
- Examine these smart technologies on application to additional cheese types and formats like soft or grated cheese, as well as other food products.
- Consider alternative forms of smart technology and their potential use with cheese products.

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APPENDIX

Survey

The purpose of this survey is to evaluate consumer attitudes towards cheese packaging, shelf-life of retail cheese products and to assess knowledge and opinions of the incorporation of additional packaging technologies within conventional cheese packaging formats.

It is important that you a consumer of cheese in order to fill in this survey.

1. *Age*

- 18 to 24
- 25 to 34
- 35 to 44
- 45 to 54
- 55 to 65
- 65 or older

2. *Gender*

- Male
- Female

3. *Please state your nationality*

4. *Highest level of education achieved.*

- Primary school or Secondary school (PS)
- Post leaving certificate course, Further education and training course, or an Apprenticeship (PFA)
- Third level certificate, Diploma, or University degree (TDU)
- Masters degree, Postgraduate diploma, Doctoral degree or Higher Doctorate (MPDH)
- No formal education

5. *Estimation of cheese consumption*

- Daily
- Weekly
- Monthly
- Rarely
- Never

6. *What type of cheese do you consume:*

- Soft
- Hard
- Both

7. *Please list the varieties of cheese you purchase most often*

8. Is the manner in which cheese is packaged important to you?

- Yes
- No

9. Score each of the following packaging features in terms of importance to you.

- *Product is contained and properly sealed*

Very Important, Somewhat Important, Not Important

- *Pack shape*

Very Important, Somewhat Important, Not Important

- *Degree of decoration or appearance*

Very Important, Somewhat Important, Not Important

- *Provision of adequate information on the label or printed on package*

Very Important, Somewhat Important, Not Important

- *Convenience features such as easy opening or resealability*

Very Important, Somewhat Important, Not Important

- *Storage, stability and shelf-life of packaged product*

Very Important, Somewhat Important, Not Important

- *Use of quality marks, symbols and icons – e.g. guaranteeing traceability or origin*

Very Important, Somewhat Important, Not Important

- *Presence of tamper evidence features or tamper-proof seals and closures*

Very Important, Somewhat Important, Not Important

- *Environmentally friendly aspects*

Very Important, Somewhat Important, Not Important

10. When you purchase cheese products, how long do you expect the product to store for?

- Days
- Weeks
- Months

11. Are you satisfied with current cheese shelf-life?

- Yes
- No

Please explain why.

12. When do you stop consuming a cheese product following purchase?

- Sell by date
- Best before date
- Use by date
- After the expiry date
- Using none of the above. Cheese acceptability decided based on sensory characteristics (e.g. appearance – the presence of mould, odour and flavour).

13. If there were safe technologies that could be used to extend the shelf-life of cheese, would you be in favour of their use?

- Yes
- No

Please explain why.

14. Would you be willing to pay more for the use of such technologies with packaged cheese products?

- Yes
- No

15. Have you heard of any of the following terms?

Smart Packaging

- Never heard of the term
- Heard of the term but do not understand it
- Heard of the term and understand the term

Active Packaging

- Never heard of the term
- Heard of the term but do not understand it
- Heard of the term and understand the term

Intelligent Packaging

- Never heard of the term
- Heard of the term but do not understand it
- Heard of the term and understand the term

Nanotechnology

- Never heard of the term
- Heard of the term but do not understand it
- Heard of the term and understand the term

16. If you have heard of any of the terms highlighted in Qu. 15 above, how did you come across these terms and was it in a positive or negative context? Please comment below.

Smart Packaging – A package that provides the consumer with an extra function beyond the basic purpose of the package (protection, containment and communication). The extra function is usually mechanical, chemical, electrical or electronic.

Active Packaging – This is a form of smart packaging. An active package contains constituents incorporated into the packaging material or within the packaging container that deliberately alter the condition of the package to either enhance sensorial properties, maintain or improve quality, or to extend the shelf-life of the product packaged.

Intelligent Packaging – This is a form of smart packaging. An intelligent package contains a device, positioned internally or externally to the package, which can monitor the condition of the product, package or package environment. The device can provide information on these aspects but does not alter the condition of the package or product.

Nanotechnology – This is the use of materials on a nanometre scale, between 1nm and 100nm in size (1nm = 1 millionth of a millimetre). Nanoparticles can expand the performance range of existing packaging materials. Particles at this size exhibit novel properties such as improved activity, mechanical, thermal, and barrier function.

17. Would you be willing to purchase a cheese product whose packaging has one or more of these technologies incorporated?

- Active Packaging only
- Intelligent Packaging only
- Nanotechnology only
- Active and Intelligent Packaging
- Active Packaging and Nanotechnology
- Intelligent Packaging and Nanotechnology
- Active Packaging, Intelligent Packaging and Nanotechnology
- None of the above

18. Would you be willing to pay more for the use of such technologies with packaged cheese products?

- Yes
- No