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Starch based systems for the colonic delivery of bioactive peptides

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Doctor of Philosophy

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Declaration

The thesis submitted is my own work and has not been submitted for another degree, either at University College Cork or elsewhere.

Signed: _____

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Abstract

Bioactive peptides have numerous health benefits, although if taken orally they may be digested during gastrointestinal (GI) transit. Encapsulation is an established method for oral delivery of bioactives. However, many current approaches arise from pharmaceutical applications and may be unsuitable for food due to the materials used, cost and scale of production. Therefore, in this project we set out to create a simple and clean-label encapsulation system, suitable for use in the food industry, which could deliver bioactive peptides to the colon. One potential clean-label entrapment material is resistant starch, which is the portion of starch that resists digestion in the upper GI tract but can be digested by bacteria in the colon. As a model bioactive peptide, the well characterised antimicrobial peptide nisin was used; this peptide is normally digested during GI transit. To prepare the nisin a simple purification process was developed, which produced a powder containing ~33% nisin from a nisin producing culture and also enriched a commercial nisin preparation over 30-fold to a purity of ~58%. The digestion of nisin was characterised (*in vitro*) and 6 nisin fragments (4 of which are bioactive) were identified in the digestion products; it was also observed that nisin formed a complex with bile salts that effected its digestion. Nisin was entrapped in starch through multiple approaches based on spray coating, co-spray drying and gel entrapment. A simple approach based on gel entrapment was the most successful and it was shown *in vitro* to be capable of protecting a portion of the entrapped nisin during transit in the upper GI tract. Using a murine model, it was determined *in vivo* that a nisin entrapped in starch gel diet significantly ($p < 0.001$, $n = 10$) affected the relative abundance of 3 times as many genera from the lower GI tract than a control nisin in starch diet, despite the mice consuming 3-fold less nisin than the control diet.

List of abbreviations

ADI	Acceptable daily intake
AFM	Atomic force microscopy
AMP	Antimicrobial peptide
AU	Arbitrary units
BSA	Bovine serine albumin
BTEE	N-benzoyl-L-tyrosine ethyl ester
CFS	Cell-free supernatant
CPP	Cell penetrating peptide
DAPI	2-(4-Amidinophenyl)-1H-indole-6-carboxamidine
DE	Dextrose equivalent
DGGE	Denaturing gradient gel electrophoresis
DIC	Differential interference contrast
DLS	Dynamic light scattering
DM	Degree of methyl-esterification
DMSO	Dimethyl sulfoxide
EC	Ethyl cellulose
EFSA	European Food Safety Authority
FDA	Food and Drug Administration
FF	Fast flow
FISH	Fluorescent in situ hybridization
FITC	Fluorescein isothiocyanate
FPLC	Fast protein liquid chromatography
GI	Gastrointestinal
GIT	Gastrointestinal tract

GRAS	Generally recognized as safe
HACS	High amylose corn starch
HPLC	High performance liquid chromatography
HPMC	Hydroxypropyl methylcellulose
HTS	High throughput sequencing
M cells	Microfold cells
MALDI TOF MS	Matrix-assisted laser desorption/ionization time of flight mass spectrometry
MD	Maltodextrin
MIC	Minimum inhibitory concentration
MOPS	3-Morpholinopropane-1-sulfonic acid
MRS	De Man, Rogosa and Sharpe
MWCO	Molecular weight cut off
NC	Nutritionally complete
PCoA	Principal coordinates analysis
PEG	Polyethylene glycol
PLA	Polylactic acid
RP-HPLC	Reversed phase - high performance liquid chromatography
RS1	Type 1 resistant starch
RS2	Type 2 resistant starch
RS3	Type 3 resistant starch
RS4	Type 4 resistant starch
sCT	Salmon calcitonin
SD	Starch dough

SD-N	Starch dough containing nisin
SEDDS	Self-emulsifying drug delivery systems
SG	Starch gel
SG-N	Starch gel containing nisin
SLN	Solid lipid nanoparticles
SNAC	Sodium N-[8-(2-hydroxybenzoyl) amino] caprylate
SWP	Supplemented whey permeate
TAT	Trans-activator of transcription
TCA	Trichloroacetic acid
TDM	Tetradecyl maltoside
TFA	Trifluoroacetic acid
WP	Whey permeate
WPI	Whey protein isolate
YE	Yeast extract
ZO-1	Zona occludens-1

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Chapter 1

Literature review: Oral delivery of bioactive proteins and peptides

1.1. Abstract

The oral route is the most acceptable way to administer therapeutic compounds; however few bioactive proteins and peptides achieve a therapeutic effect when ingested. There are two major challenges in oral delivery of most bioactive proteins and peptides; when transit through the gastrointestinal tract (GIT) is required the challenge is protection from digestion and when systemic delivery is required the challenge is absorption. A range of strategies to enable the oral delivery of proteinaceous bioactives are discussed in this review, including the use of pro-peptides, structural modification of the protein/peptide, protease inhibitors, absorbance/permeation enhancers, bioadhesive systems, emulsion/lipid based systems, nanoparticle based systems and controlled release systems. Despite the range of tools available to enable oral delivery, there are relatively few systems in commercial use or in commercial development, for applications in food or pharmaceutical products. The diversity between commercial systems designed for the same or similar proteins and peptides shows that clear optimal systems for oral delivery of proteins and peptides are yet to be developed, thus there is much potential for further research in this field.

1.2. Introduction

Multiple studies have shown that the oral delivery of bioactives is preferred over more invasive routes such as injection (Stewart et al., 2016). However, few bioactive proteins and peptides are orally delivered effectively; only 4% of FDA approved therapeutic peptides are orally administered, with the majority being

administer intravenously, subcutaneously or intramuscularly (45%, 33%, and 14% respectively) (Usmani et al., 2017). To avail of their health benefits after ingestion, many bioactive proteins and peptides require delivery to a specific location in the GIT, as in the case of the colonic delivery of ciclosporin (Keohane, Rosa, Coulter, & Griffin, 2016), or they require absorption into the systemic circulation, as in the case of insulin (Oramed Pharmaceuticals, 2018). Therefore there are two primary challenges for the oral delivery of most bioactive proteins and peptides, namely protection from pH and proteases during transit through the GIT and absorption from the GIT. Both of these challenges do not occur in all proteins and peptides; a peptide can have a local activity in the GIT or their site of absorbance could be the mouth (Bernstein, 2008; Fretzen et al., 2016).

Some bioactive peptides are stable at low pH and have an inherent resistant to digestion, such as those in which cleavage sites for particular enzymes are absence or inaccessibility, as in the case of isoleucine-proline-proline (IPP) (FitzGerald & Meisel, 2000) and plecanatide (Pitari, 2013). However, the vast majority of bioactive peptides are susceptible to digestion during gastro-intestinal transit (Segura-Campos, Chel-Guerrero, Betancur-Ancona, & Hernandez-Escalante, 2011), including peptides with significant potential health benefits such as insulin (Sonia & Sharma, 2014) and pediocin (Kheadr et al., 2010).

Protein/peptide digestion begins in the stomach; gastric acid denatures and unfolds most proteins, thus allowing proteolytic enzymes better access to their respective cleavage sites. The protease pepsin is secreted in the stomach and is capable of cleaving 10-15% of the peptide bonds depending on the ingested proteins/peptides. The majority of protein/peptide digestion occurs in the small intestine, primarily due to proteases produced by the pancreas, which are secreted

into the small intestine (trypsin, chymotrypsin, elastase and carboxypeptidases A and B). Additionally there are aminopeptidases located on the surface (brush border) of the epithelium (Goodman, 2010).

Proteinaceous materials are absorbed through enterocytes (intestinal absorptive cells) that are located in the epithelium of the small intestine. The uptake mechanisms are designed for amino acids, dipeptides and tripeptides. Without resorting to absorbance enhancers or cell penetrating peptides, there are limited possibilities for the uptake of larger peptides; primarily via the antigen sampling mechanism of microfold cells (M cells) and by passive diffusion of highly lipid-soluble peptides into enterocytes (Miner-Williams, Stevens, & Moughan, 2014).

Carbohydrate digestion and absorption in humans (excluding the GIT microbiota) is essentially limited to simple sugars, digestible dextrins, digestible starches and glycogen (Lunn & Buttriss, 2007). Carbohydrate digestion begins in the mouth with the digestion of complex carbohydrates with salivary α-amylase, which is subsequently deactivated by the acidic pH of the stomach. Carbohydrate digestion continues in the small intestine with α-amylase that is produced by the pancreas. This cleaves complex carbohydrates into di-, tri-, and oligosaccharides. Some dietary carbohydrates such as the disaccharides sucrose and lactose do not require α-amylase digestion. The di-, tri-, and oligosaccharides are broken down to monosacharides by brush border enzymes and then absorbed by the enterocytes (Goodman, 2010).

Digestion of lipids begins in the mouth with lingual lipase and continues in the stomach with lingual lipase and gastric lipase, although only 15% of dietary lipids are digested at these stages. In the small intestine bile salts emulsify the lipids while pancreatic lipase and co-lipase work in conjunction to digest lipids. The products of lipolysis are transported by bile salt micelles to the enterocytes

(Goodman, 2010; Minekus et al., 2014). An overview of protein/peptide, carbohydrate and lipid, digestion and absorption, is given in Fig. 1.1.

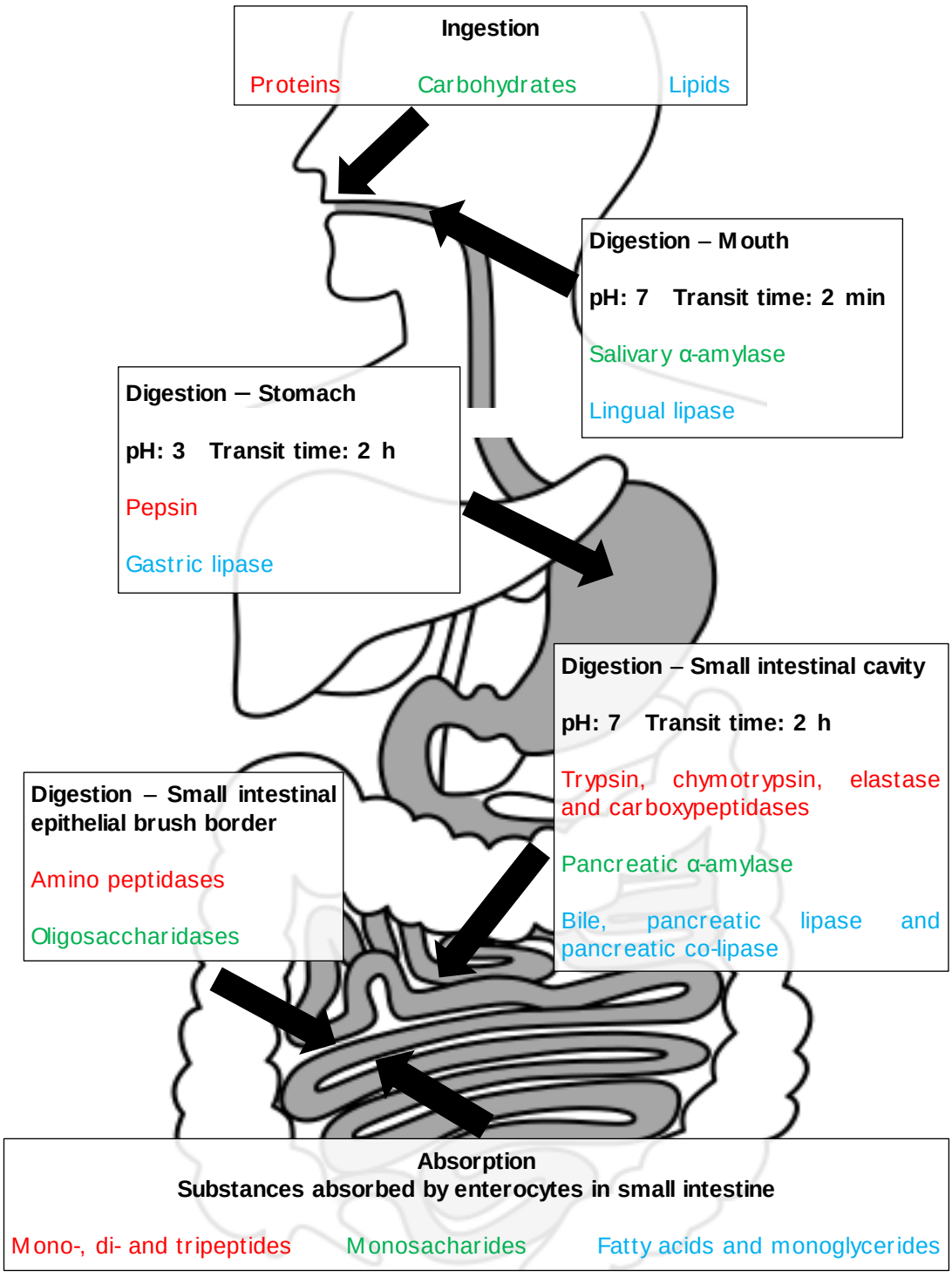

Fig. 1.1. An overview of protein/peptide, carbohydrate and lipid digestion and absorption. Based on Minekus et al. (2014) and Goodman (2010).

The difference between proteins and peptides is purely one of size; generally molecules with more than 50 amino acid residues are referred to as proteins, however there is no clear-cut definition with bioactives such as insulin being referred to as a peptide and as a protein (Doonan, 2002). For brevity the word peptide will be used in this review to refer to proteins and peptides, as the majority of proteinaceous bioactives mentioned in this review are smaller than 50 amino acids.

The oral delivery of bioactive peptides is a challenge for both the food and the pharmaceutical industries (Fosgerau & Hoffmann, 2015; Lau & Dunn, 2017; Mohan et al., 2015). The development of oral delivery systems for bioactive peptides dates to at least the 1920s, when studies on oral insulin delivery were performed (Harrison, 1923). The treatment of diabetes continues to be one of the main drivers of research in oral peptide delivery, with several systems in commercial development for the oral delivery of insulin (Diabetology, 2017a; Diasome Pharmaceuticals, 2017; Oramed Pharmaceuticals, 2018; Pozzilli, Raskin, & Parkin, 2010).

The primary strategies employed in the oral delivery of bioactive peptides are pro-peptides, structural modification of the peptide, protease inhibitors, absorbance/permeation enhancers, bioadhesive systems, emulsion/lipid based systems, nanoparticle based systems and controlled release systems.

1.3. Delivery systems

1.3.1. Pro-peptides

The pro-peptide strategy involves ingesting a protein or peptide whose subsequent digestion in the gastrointestinal tract will produce peptides that are

bioactive; this can occur during the digestion of many common foodstuffs such as milk (Hartmann & Meisel, 2007). The antimicrobial peptide lactoferricin, which is active against Gram positive and negative bacteria, is produced during the digestion of the milk protein lactoferrin by pepsin in the stomach (Gifford, Hunter, & Vogel, 2005). The antioxidant LVGDEQAVPAVCVP has been produced through the (*in vitro*) gastric and small intestinal digestion of mussels (Jung et al., 2007), while antihypertensive peptides ER and RPR were produced through the (*in vitro*) gastric and small intestinal digestion of pork (Escudero, Sentandreu, Arihara, & Toldra, 2010).

The antihypertensive lactotripeptides isoleucine-proline-proline (IPP) and valine-proline-proline (VPP) are produced by both processing and gastrointestinal digestion of milk proteins (Boelsma & Kloek, 2009). They have received much interest, as in addition to being sufficiently short to be absorbed normally, they have significant resistance to digestion as they have no cleavage sites for pepsin, trypsin, chymotrypsin or elastase (Goodman, 2010) and their C-terminal Pro-Pro gives them a general resistance to digestive enzymes including proline specific peptidases (FitzGerald & Meisel, 2000). However, a review by the European Food Safety Authority (EFSA) of 25 human intervention studies concluded there was no evidence of an effect of IPP and VPP on blood pressure (EFSA panel on dietetic products nutrition and allergies, 2012).

1.3.2. Structural modification

The linking of a peptide to one or more polyethylene glycol (PEG) chains is termed PEGylation (Veronese & Pasut, 2005). The PEGylation of peptides such as

calcitonin, glucagon-like peptide-1 (GLP-1) and insulin has been associated with greater proteolytic resistance and systemic retention without a negative impact on absorption or activity (Calceti, Salmaso, Walker, & Bernkop-Schnurch, 2004; Chae et al., 2008; Youn et al., 2006).

Replacing L-amino acids amino acids with their enantiomers (D-amino acids) increases the proteolytic resistance of a peptide (Feng & Xu, 2016), an example of which is the antiduretic desmopressin (Cvetkovic & Plosker, 2005).

Cyclisation of peptides increases proteolytic resistance by making the N and C terminals, which are normally targets for proteolysis, less accessible for exopeptidases; additionally cyclisation can allow stabilisation of conformations in which polar residues are exposed, making the molecule more lipid soluble, thus allowing absorption by passive diffusion, as in the case of cyclosporine (Nielsen et al., 2017). Methods used in producing cyclic peptides include creating an amide bond between the N and C terminals of the peptide and forming bonds such as disulfide, lactam, lactone and ether bridges between the side change of amino acids (Li & Roller, 2002).

The B_{12} absorption pathway can be used to absorb peptides by conjugating the peptides to B_{12}. This has been successfully used to enhance the absorbance of insulin (Clardy-James, Allis, Fairchild, & Doyle, 2012). However this approach is limited by the quantity of B_{12} that can be absorbed (1 to 1.5 μg/day) (Marie Sych, Lacroix, & Stevens, 2016) and needs to be performed in conjunction with a strategy to prevent proteolysis (Petrus, Fairchild, & Doyle, 2009).

1.3.3. Enzyme inhibitors

The mechanism that inhibitors use to inhibit the action of a protease include binding to the protease such that its structure is distorted, binding to the protease in the place of the substrate or depriving the protease of an essential co-factor such as a metal ion (Bernkop-Schnurch, 1998; Otlewski, Jelen, Zakrzewska, & Oleksy, 2005). A large range of inhibitors have been trialled for peptide delivery, ranging from traditional inhibitors such trypsin soybean inhibitor, Bowman-Birk inhibitor (BBI) and elastatinal to more recently characterised inhibitors such as ovomucoids (Agarwal, Nazzal, Reddy, & Khan, 2001; Laskowski et al., 1958; Marschutz & Bernkop-Schnurch, 2000; Tozaki et al., 1997). There are safety concerns regarding the side effects of inhibitors including disruption of the digestion of nutritive proteins and the stimulation of protease secretion caused by a feedback regulation, which results in hypertrophy and hyperplasia of the pancreas and ultimately cancerous tumours with prolonged use of inhibitors (Bernkop-Schnurch, 1998).

1.3.4. Absorption/permeation enhancers

Absorption enhancers used in the delivery of peptides include bile, (Michael et al., 2000), chitosan (Thanou, Verhoef, & Junginger, 2001), cell permeating peptides (Morishita et al., 2007), fatty acids (Leonard, Lynch, McKenna, & Brayden, 2006; Maher, Leonard, Jacobsen, & Brayden, 2009), surfactants (Alama et al., 2016) and chelating agents (Morishita et al., 1993) including citric acid (Welling et al., 2014).

Despite differences in the initial effect, such as chelating agents depleting intracellular calcium and chitosan drawing the zona occludens-1 (ZO-1) and occludin into the cytoskeleton, most of the absorption enhancers ultimately achieve

their function through the opening of the epithelial tight junctions (Alama et al., 2016; Leonard et al., 2006; Maher et al., 2009; Michael et al., 2000; Morishita et al., 1993; Smith, Wood, & Dornish, 2004; Thanou et al., 2001; Welling et al., 2014). Other absorption mechanisms used for peptide delivery include increasing membrane fluidity using surfactants and inducing macropinocytosis using cell penetrating peptides (CPPs) (Alama et al., 2016; Morishita et al., 2007).

The concentration of the absorption enhancer needs to be carefully adjusted to strike a balance between the degree of absorption and toxicity; also there is always the risk of unwanted molecules being absorbed alongside the molecule of interest (Aungst, 2012; Whitehead, Karr, & Mitragotri, 2008).

One approach being used to circumvent the issue of nonspecific absorbance is directly conjugating a nutrient such as vitamin B_{12} or B_7 (biotin) to the peptide or the peptide containing delivery system, which are then able to take advantage of the absorbance system for that nutrient (Petrus et al., 2009; Zhang et al., 2014).

1.3.5. Bioadhesive systems

These systems increase the residence/contact time of the peptide at the site of absorption; which increases the amount absorbed. Examples include those using lectins, polyacrylates and polysaccharides such as chitosan and hydroxyethyl cellulose, with adhesion occurring to the mucus layer or the epithelial cells (Bernkop-Schnurch & Krajicek, 1998; Gabor, Schwarzbauer, & Wirth, 2002; Grabovac, Guggi, & Bernkop-Schnurch, 2005; Zhang et al., 2006).

Much of the recent bioadhesive research has been on thiomers which are produced by addition of a thiol group bearing side chains to polymers such as

chitosan. They are capable of forming covalent bonds with the cysteine-rich subdomains of mucus glycoproteins, which is a stronger interaction than conventional bioadhesives (Bernkop-Schnurch, 2005; Bonengel & Bernkop-Schnurch, 2014).

1.3.6. Protective matrices/coatings/carriers with controlled release

A peptide can be protected from digestion during GIT transit by entrapping it within a protective matrix. However this matrix could inhibit peptide activity and absorption. Therefore a mechanism for releasing the peptide from the matrix is required such as release that is triggered due to pH (pH release systems), release that happens continually at a controlled rate (time release systems) or release due to digestion by colonic bacteria (microbial release systems).

The matrices in a pH release system are formulated to swell or dissolve at a particular pH, thus releasing the peptide. Natural polymers used in pH release system include alginate, chitosan and shellac (George & Abraham, 2007; Jing et al., 2017; Kraisit et al., 2013). The most commonly studied polymer for pH release system is methacrylate and its derivatives including poly(methacrylic acid-g-ethylene glycol) (Kamei et al., 2009; Tuesca, Reiff, Joseph, & Lowman, 2009) and copolymers of 2-hydroxyethyl methacrylate and methacrylic acid (Mahkam, 2005), with a particular interest in the commercial Eudragit® range (Jain, Panda, & Majumdar, 2005; Marais et al., 2013)

The difference between the acidic pH in the stomach and neutral pH in the small intestine makes it possible to use pH triggered release to target the small intestine (Wang & Zhang, 2012). However, targeting of the colon with a pH

triggered release is more challenging as in the ileum of the small intestine the pH reaches pH 7.5 , then in the caecum/ascending colon it drops to pH 6.4 and then it rises along the colon to pH 7.0 in the descending colon, additionally there are person to person variations in these values (Evans et al., 1988). Due to this, delivery systems for the colon with a pH based release are normally designed so that they begin releasing in the ileum of the small intestine and are often referred to as ileo-colonic delivery systems instead of colonic delivery systems (McConnell, Short, & Basit, 2008); this results in exposure to the proteases of the small intestine. An additional difficulty regarding pH based ileo-colonic release is that the delivery system is not exposed to pH > 7 for long enough to achieve release in a portion of individuals (Ibekwe et al., 2008; Ibekwe et al., 2006).

Time release systems are normally based on a matrix that can swell in an aqueous solution. By altering the composition of the matrix including the degree of crosslinking and thickness, the rate of swelling and thus the rate of peptide release can be controlled. The natural polymers chitosan (Yuan, Jacquier, & O'Riordan, 2018) and alginate (Liu, Chen, Xie, & Zhang, 2003) have been used in systems for the colonic delivery of insulin and bee venom peptide respectively. In gels composed of cross-linked alginate and chitosan, the ratio of alginate to chitosan determined the rate of release of bovine serine albumin (BSA) (Xu et al., 2007). Time release systems for the colonic delivery of insulin have also been produced based on hydroxypropyl methylcellulose (HPMC) and polymethacrylate (Del Curto et al., 2011; Maroni et al., 2016).

Time release systems have been combined with pH release systems (Del Curto et al., 2014; Liu et al., 2003). This approach is of particular use in colonic

delivery, as by having an outer pH release coating, time release can be delayed until the small intestine, which allows a greater portion of the peptide to reach the colon.

There are approximately 4×10^{13} bacteria in the colon (Sender, Fuchs, & Milo, 2016) and these consume what escapes digestion or is indigestible in the upper gastrointestinal tract (Chassard & Lacroix, 2013). This fact allows for the possibility of using microbial release systems for colonic delivery, based on materials that are not digestible in the upper gastrointestinal tract but can be digested by colonic bacteria.

There was originally much interest in azopolymer based microbial release systems for colonic delivery (Saffran et al., 1986; Tozaki et al., 2001), however there are recent concerns that the metabolism of these can result in potentially carcinogenic by-products (Claus, Guillou, & Ellero-Simatos, 2016).

Safer microbial release systems for colonic delivery of peptides have therefore been developed based on polymers of commonly used and food-grade polysaccharides including chitosan, pectin and starch (Atyabi, Inanloo, & Dinarvand, 2005; Fetih et al., 2006; Pu et al., 2011; Zhang, Alsarra, & Neau, 2002).

Chitosan is prepared by the deacetylation of chitin, a structural biopolymer found in the exoskeleton of arthropods and in the cell walls of fungi and yeast (Pillai, Paul, & Sharma, 2009). This is necessary as chitin is insoluble in almost all common solvents, whereas chitosan is soluble below pH 6 (Pillai et al., 2009). Solid gels are primarily produced through using a crosslinking agent such as glutaraldehyde (Mirzaei, Ramazani, Shafiee, & Danaei, 2013) or through ionic interactions with an oppositely charged polymer such as pectin (Chen et al., 2010). Chitosan has the additional advantages that it is mucoadhesive (Grabovac et al., 2005) and an absorbance enhancer (Smith et al., 2004). Peptides incorporated into chitosan based

colonic delivery systems include insulin (Yuan et al., 2018) and calcitonin (Fetih et al., 2006).

Pectins are a mixture of polysaccharides that are found in plant cell. For pectins the procedure required to create a solid gel depends on their degree of methyl-esterification (DM), which in turn depends the source of the pectin (Sriamornsak, 2003). High DM (>60%) pectins form gels at pH 2.8 to 3.5 and high soluble solids, whereas low DM (<40%) pectins require calcium or another divalent cation for gelation (Belitz, Grosch, & Schieberle, 2009; Sriamornsak, 2003). Pectin has been used for the colonic delivery of nisin (Ugurlu, Turkoglu, Gurer, & Akarsu, 2007) and insulin (Cheng & Lim, 2004).

Starch is comprised of the glucose polymers amylose and amylopectin. Both amylose and amylopectin are comprised of linear chains of glucose linked by α-1,4 glycosidic bonds and these chains are branched by α-1,6 glycosidic bonds (Fig. 1.2). Amylose is infrequently branched while amylopectin is branched at 10 nm intervals. In its native form, starch is arranged into semi-crystalline starch granules, which are of 0.1 to 200 μm in diameter. If these granules are heated in the presence of water, the amylose and amylopectin dissociate, which leads to the granule exuding amylose and absorbing water, causing them to swell and ultimately dissipate. Subsequent cooling of the solution causes the amylose and amylopectin to re-associate, turning the solution into a solid gel. These two stages are referred to as gelatinisation and retrogradation (Fig 1.3). The strength of the gel is primarily related to its amylose content (Alcázar-Alay & Meireles, 2015; Wang, Li, et al., 2015).

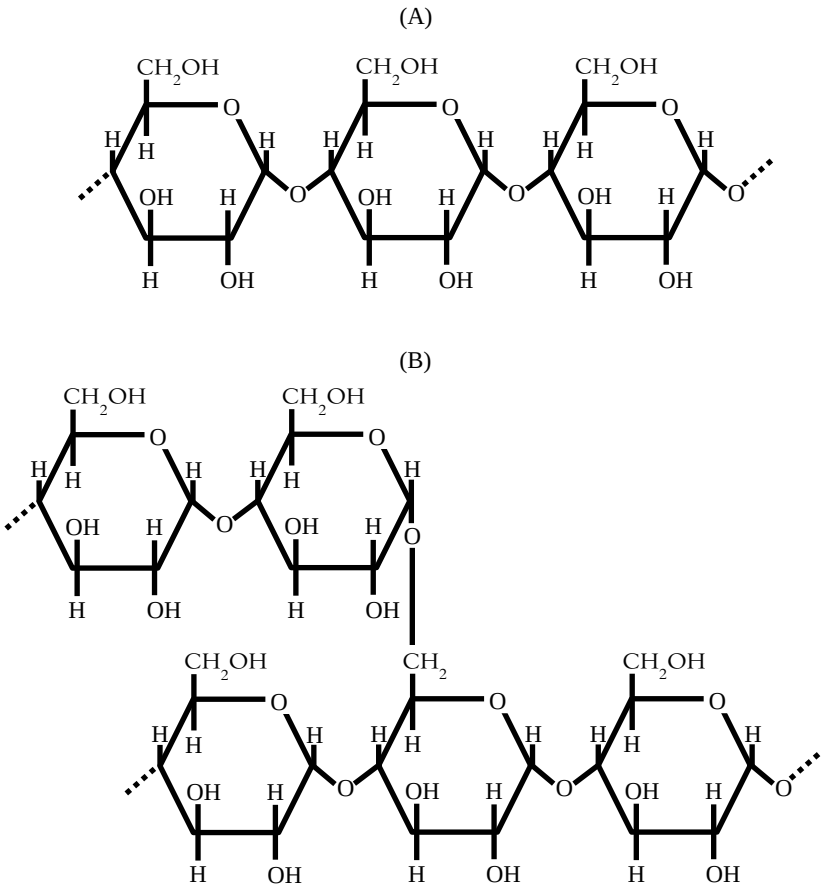

Fig. 1.2. Structure of amylose (A) and amylopectin (B).

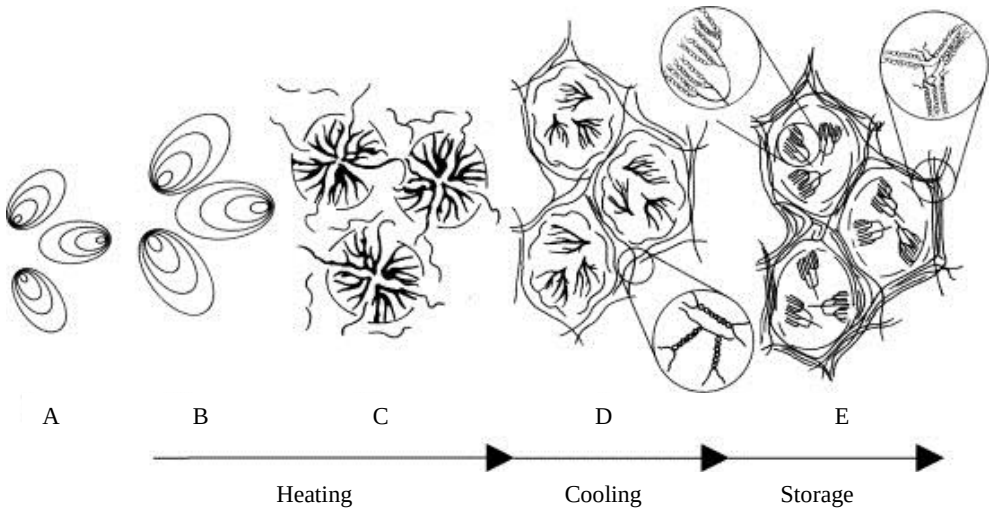

Fig. 1.3. Schematic representation of starch gelatinisation and retrogradation. The stages are as follows: native starch granules (A), swelling of granules during initial gelatinisation (B), amylose leaching and disruption of starch granules during further gelatinisation (C), re-association of amylose during initial retrogradation (D) increased association of amylose and re-association of amylopectin during further retrogradation. Image obtained from Goesaert et al. (2005).

The portion of starch that resists digestion in the small intestine but can be fermented by the colonic microbiota is termed ‘resistant starch’ and varies between starch source and type (Bird, Lopez-Rubio, Shrestha, & Gidley, 2009). Resistant starch is classified into 4 types: type 1 resistant starch (RS1) is resistant due to physically inaccessibility, type 2 resistant starch (RS2) is resistant due to being in a granular form, type 3 resistant starch (RS3) is resistant due to retrogradation and type 4 resistant starch (RS4) is resistant due to chemical modification (Sajilata, Singhal, & Kulkarni, 2006).

When starch (or its component polymers) are used as a protective coating for colonic delivery, they are normally combined with a binder/plasticizer to control swelling in aqueous solution and increase structural integrity, with the most studied being ethyl cellulose (Freire et al., 2010; McConnell et al., 2007). Peptides that have been incorporated into starch based colonic delivery systems include hepatocyte growth factor (Pu et al., 2011) and insulin (Situ, Chen, Wang, & Li, 2014), with triacetin and 1,2-propanediol as their respective binder/plasticizer.

1.3.7. Nanoparticles

A nanoparticle is a particle that is measured on a nanometre (nm) scale and usually refers to particles below 1 μm (Buzea, Pacheco, & Robbie, 2007). The primary advantage of nano-sized delivery systems for peptides is their capacity to pass through the mucus and epithelial barriers, particularly if the appropriate absorbance/permeation enhancers are affixed on their surface (Date, Hanes, & Ensign, 2016). There are a large range of materials (including polylactic acid (PLA), polyethylene glycol (PEG), chitosan and alginate) and techniques (including

emulsification-solvent evaporation, interfacial polymerization and supercritical fluid technology) being investigated to produce nanoparticles for peptide delivery, with the optimum material and technique dependent on both the properties of the peptide and the desired properties of the product (Reis, Neufeld, Ribeiro, & Veiga, 2006).

Initial protection during GIT transit is provided using the approaches described previously such as by PEGylation, protease inhibitors and coatings with pH mediated dissolution (Date et al., 2016; He et al., 2017; Malhaire et al., 2016; Zhang et al., 2017).

To limit impedance by the mucus layer two common approaches used are having a particle surface whose net charge is neutral and coating the surface with low molecular weight PEG. To transverse the epithelial cell layer there has been some success using conventional absorbance enhancers incorporated in the nanoparticle, particularly CPPs; however the approaches yielding the most interest are exploitation of the endocytosis and transcytosis systems (Lundquist & Artursson, 2016; Malhaire et al., 2016). Incorporation of vitamin B_{12} onto the surface of insulin loaded nanoparticles allowed them to use the B_{12} transport system and be endocytosed by intestinal enterocytes (Chalasani et al., 2007). One particularly promising transcytosis approach is incorporating Fc into the surface of the nanoparticles, which results in them being transported across the intestinal epithelium (Pridgen et al., 2013). It is also possible for nanoparticles to be taken up by the M cells in the Peyer's patches in the same manner as any antigen entering the body, however, while some authors have highlighted the potential of this approach, others have claimed that there are insufficient M cells in the body to make this an efficient uptake route (Date et al., 2016; Lundquist & Artursson, 2016; Malhaire et al., 2016).

1.3.8. Lipid based systems including emulsions

Although lipids are not a specific system per se, their versatile properties give them a functional role in a wide range of systems. Lipids allow the solubilisation of hydrophobic peptides such as calcitonin and cyclosporine (Aguirre, Rosa, Coulter, & Brayden, 2015; Choc, 1997).

The rate of peptide release is determined by the rate of digestion of the emulsion by lipolysis. The longer the chain length of the fatty acids in an emulsion, the greater the resistance of the emulsion to lipolysis. Therefore the rate of release can be controlled through the composition of the emulsion (Giroux, Robitaille, & Britten, 2016). There are many ways that lipids can enhance the uptake of peptides; the fatty acid derivatives caprylate and caprate can dilate tight junctions (Leonard et al., 2006; Maher et al., 2009), nanoparticles made from lipids such as phosphatidylcholine can be absorbed into a cell by endocytosis and passive diffusion (Rao, Agarwal, & Shao, 2008) and by complexing a peptide with sodium N-[8-(2-hydroxybenzoyl) amino] caprylate (SNAC) a lipophilic complex can form which is capable of transcellular absorption (Prem Victor, Paul, & Prakash Sharma, 2014).

It is possible to incorporate hydrophilic peptides such as insulin in a lipid system through water in oil (w/o) or water in oil in water (w/o/w) double emulsions. (Cardenas-Bailon, Osorio-Revilla, & Gallardo-Velazquez, 2015; Li et al., 2017; Mutaliyeva et al., 2017).

Self-emulsifying systems, commonly described as self-emulsifying drug delivery systems (SEDDS), are mixtures of peptides, lipids and surfactants that once dispersed in an aqueous solution and mildly agitated, form emulsions (Kohli et al.,

2010). The emulsion droplets are commonly nano-sized (Kohli et al., 2010). Two commercial oral delivery systems (NeOral® and SmPill®) use SEDDS for the delivery of ciclosporin (Keohane et al., 2016; Ritschel, 1996). By altering the composition of the SEDDs and by the choice of excipients it is possible to modulate the rate of digestion by lipase, reduce mucus impediment, enhance epithelial absorption and control the rate of release (Leonaviciute & Bernkop-Schnurch, 2015).

Two other lipid based techniques that have attracted interest are solid lipid nanoparticles (SLN) and liposomes. SLN are distinguished by being nano-sized particles that are composed of lipids that are solid at room and body temperature; this allows greater control over peptide release compared to other lipid systems (Geszke-Moritz & Moritz, 2016).

Liposomes are comprised of an aqueous core that is enclosed by a lipid bilayer. In their native state they have low stability during gastrointestinal transit (Wu, Lu, & Qi, 2015) and recent studies using them for oral peptide delivery have examined how their stability and absorption can be improved by incorporating excipients. Incorporating biotin into the membrane of nisin containing liposomes increased their *in vivo* intestinal absorption compared to the controls, due to the biotin inducing endocytosis in enterocytes (Zhang et al., 2014). Liposomes coated with carbopol, chitosan or thiomers had enhanced mucoadhesiveness which increased the *in vivo* or *ex vivo* absorption of calcitonin containing liposomes (Gradauer et al., 2013; Takeuchi, Matsui, Yamamoto, & Kawashima, 2003). Incorporating bile salts into liposomes that contained insulin increased their oral bioavailability in rats by protecting the liposomes from physiological bile salts (Niu et al., 2012). Diasome Pharmaceuticals are currently developing a liposome based system for oral insulin delivery (Geho, Geho, Lau, & Gana, 2009).

1.4. Commercial oral peptide delivery systems

Fig. 1.4. A selection of commercially available orally delivered bioactive peptides. From left to right and top to bottom: ciclosporin (Neoral®, Novartis), pancreatic enzymes (CREON®, Abbott), plecanatide (Trulance™, Synergy Pharmaceuticals), lunasin (LunaRich® Reliv), vancomycin (Firvanq™ Cutispharma), desmopressin (Minirin®, Ferring Pharmaceuticals), linaclotide (Linzess®, Ironwood Pharmaceuticals in collaboration with Allergan) and tyrothricin (Lemocin®, Gebro Pharma).

An overview of orally delivered bioactive peptides that are commercially available (Fig. 1.4) or approaching commercialisation are listed in Table 1.1. In cases where several manufactures produce the same peptide with the same delivery approach, only one manufacturer is listed for brevity.

Many systems depend on peptides that have structural properties that make them suitable for colonic delivery such as being cyclic and/or containing D amino acids; this severely limits the number of peptides for which these systems would be suitable.

The approaches that do not depend on the structural properties of the peptide tend to use complex delivery systems which combine several strategies such as an outer coating with controlled release, protease inhibitors and absorbance enhancers into a single system. This complexity may make such a system only suitable for a high value product, such as pharmaceutical products or supplements.

Despite the structural similarity of many peptides in Table 1.1 and the frequent goal of absorption in the small intestine, a different delivery approach is used for each peptide; even the Eligen® system which is used for both calcitonin and semaglutide uses a different absorbance enhancer for each peptide (5-CNAC for calcitonin and SNAC for semaglutide (Davies et al., 2017; Karsdal et al., 2011). The range of approaches being investigated, even at a commercial stage, shows that the field is highly dynamic, constantly developing and there is still much research required before standard delivery systems suitable for a range of peptides are be achieved.

Finally it is notable that there is apparently only one colonic targeting system being commercially developed (SmPill® (Keohane et al., 2016)); thus it is an area ripe for further research.

Table 1.1. Orally delivered bioactive peptide systems that are commercially available or in commercial development.

Peptide	Brand Delivery system Company	Condition being treated	Development Stage	Target	Delivery system	References
Calcitonin (salmon calcitonin (sCT))	Eligen® Emisphere Technologies	Osteoporosis	Phase 3 clinical trial completed.	Absorption in small intestine.	Protease inhibitor that also functions as an absorbance enhancer.	Karsdal et al. (2011) Emisphere Technologies (2018)
Calcitonin (salmon calcitonin (sCT))	TBRIA™ Peptelligence® Colaberation between Enteris BioPharma and Tarsa Therapeutics	Osteoporosis	Phase 3 clinical trial completed.	Absorption in small intestine.	Protective outer coating with pH controlled release. Protease inhibitor that also functions as an absorbance enhancer.	Enteris BioPharma (2015) Binkley et al. (2012)
Ciclosporin	Neoral® Novartis	Transplant rejection	Commercially available.	Absorption in small intestine.	Cyclic peptide in self emulsifying nano sized particles.	Drewe, Beglinger, and Kissel (1992) Gibaud and Attivi (2012) Choc (1997)
Ciclosporin	SmPill® Sigmoid Pharma	Ulcerative colitis	Phase 2 clinical trials completed.	Absorption in colon (peptide remains locally).	Protective outer coating with time controlled release. Cyclic peptide in self emulsifying nano sized particles.	Keohane et al. (2016) Sigmoid Pharma (2017)
Desmopressin	Minirin® Ferring Pharmaceuticals	Nocturia	Commercially available	Absorption in upper small intestine.	Chemical modification including an L to D amino acid substitution.	White and Bradnam (2015) Cvetkovic and Plosker (2005)
Insulin	Capsulin™ Axcess™ Diabetology	Diabetes	Phase 2 clinical trials are ongoing.	Absorption in upper small intestine.	Protective outer coating with pH controlled release. Protease inhibitors and absorbance enhancer.	Diabetology (2017a) Diabetology (2017b)
Insulin	Hepatic-directed vesicle-insulin (HDV-I) Diasome Pharmaceuticals	Diabetes	Phase 2 clinical trials completed.	Absorption in small intestine.	Liposome based nanoparticle with absorbance enhancer.	Geho et al. (2014) Diasome Pharmaceuticals (2017) Geho et al. (2009)
Insulin[2]	Oral-Lyn™ Generex Biotechnology	Diabetes	Phase 3 clinical trials are ongoing.	Absorption in buccal mucosa.	Absorbance enhancers.	Pozzilli et al. (2010) Bernstein (2008)

Insulin	ORMD-0801 POD™ Oramed Pharmaceuticals	Diabetes	Phase 2 clinical trials completed.	Absorption in small intestine.	Protective outer coating with pH controlled release. Protease inhibitors and absorbance enhancer.	Oramed Pharmaceuticals (2017) Oramed Pharmaceuticals (2018)
Lunasin	LunaRich® Reliv	Heart disease (excessive cholesterol) Under investigation as a cancer treatment	Commercially available.	Absorption in small intestine.	Protease inhibitors. The peptide has a 3 amino acid motif that induces macropinocytosis.	Lule et al. (2015) Park, Jeong, and Lumen (2007) Reliv (2018a) Reliv (2018b)
Linaclotide	Linzess® Collaboration between Ironwood Pharmaceuticals and Allergan	Irritable bowel syndrome (IBS)	Commercially available.	Small and large intestine (not absorbed).	Protective outer coating with pH controlled release. Cyclic peptide.	Parker, Yuan, and Liu (2013) Fretzen et al. (2016) Ironwood Pharmaceuticals and Allergan (2018)
Octreotide	Mycapssa® Chiasma	Acromegaly	Phase 3 clinical trials are ongoing.	Primarily absorbed in small intestine.	Protective outer coating with pH controlled release. Cyclic peptide which possess amino acids in D configuration in lipid with absorbance enhancer.	Tuvia et al. (2014) Chiasma (2018) Wang, Yadav, et al. (2015)
Pancreatic enzymes	CREON® Abbott	Exocrine pancreatic insufficiency	Commercially available.	Small intestine (not absorbed).	Protective outer coating with pH controlled release.	Kuhn, Gelrud, Munck, and Caras (2010)
Plecanatide	Trulance™ Synergy Pharmaceuticals	Chronic idiopathic constipation (CIC)	Commercially available.	Proximal small intestine (not absorbed).	Cyclic peptide that has pH stability (although its activity is pH dependent).	Pitari (2013) Miner (2018) Al-Salama and Syed (2017)
Semaglutide	NN9924 Eligen® Collaboration between Novo Nordisk and Emisphere	Diabetes	Phase 3 clinical trials are ongoing.	Absorbed in stomach.	Protease inhibitor that also functions as an absorbance enhancer.	Davies et al. (2017) Novo Nordisk (2018)
Taltirelin	Ceredist® Mitsubishi Tanabe Pharma	Spinocerebellar ataxia	Commercially available.	Absorbed in small intestine.	Structurally modified. As a tripeptide normal absorption is possible.	Khomane, Meena, Jain, and Bansal (2011) Kinoshita et al. (1998)

Tyrothricin (this is a group of closely related peptides)	Lemocin® Gebro Pharma	Throat infections	Commercially available.	Throat (not absorbed).	Primarily comprised of cyclic peptides with a number of amino acids in D configuration.	Lang and Staiger (2016) Gebro Pharma (2018)
Vancomycin	Firvanq™ Cutispharma	*Clostridium difficile*-associated diarrhea Enterocolitis caused by *Staphylococcus aureus*	Commercially available.	Small and large intestine (not absorbed).	Cyclic glycopeptide.	Levine (2006) Cutispharma (2018)

1.5. Conclusions

The lack of systems for the oral delivery of bioactive peptides limits the potential health benefits of these peptides, particularly those that could be applied locally in the GIT. There is a large range of tools that can aid oral delivery of bioactive peptides, however very few delivery systems, especially colon targeted systems, that reach commercial development. There exists a need to develop oral delivery systems, particularly ones that are simple to produce yet suitable for a range of peptides, to take advantage of the full potential of bioactive peptides in the future.

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Chapter 2

A simple method for the purification of nisin

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2.1. Abstract

Nisin, an antimicrobial peptide showing activity against a broad range of Gram positive bacteria is widely used as a food preservative and has potential as a therapeutic for a range of infectious diseases. Here, we present a simple purification method, based on a salting-out approach, which can produce a powder containing ~33% nisin, from a nisin-producing culture in a whey permeate-based medium. This process removes over 99% of the lactic acid, NaCl, lactose and non-nisin proteins from the cell-free culture supernatant. The approach can also enrich a commonly used commercial nisin preparation over 30-fold to a purity of ~58%. These are higher purities than comparable published methods. The simplicity of this approach facilitates its use in research and also its scale-up.

2.2. Introduction

Nisin is an antimicrobial peptide produced by strains of *Lactococcus lactis* subsp. *lactis* (Abee & Delves-Broughton, 2003). Nisin has been granted Generally Recognized As Safe (GRAS) status by the US Food and Drug Administration (FDA) (US Food and Drug Administration, 1988) and is widely used as a food preservative (Abee & Delves-Broughton, 2003) in foodstuffs ranging from cheese and soups to beer and sausages (Delves-Broughton, 2005). It is produced by a range of companies including DuPont under the brand name Nisaplin®, DSM under the brand name Delvo®Nis and Chr. Hansen under the brand name Chrisin™. Nisin has been investigated as a treatment for a range of infections (De Kwaadsteniet, Doeschate, & Dicks, 2009; Heunis, Smith, & Dicks, 2013; Le Lay et al., 2016), as a cancer therapy

(Kamarajan et al., 2015) and proposed as a growth supplement for poultry (Józefiak et al., 2013). Nisin has the greatest solubility and stability at pH 3-3.5, with solubility and stability decreasing with increasing pH (Davies et al., 1998; Rollema et al., 1995). Heating nisin to 115 °C for 20 min at pH 3 results in less than 5% loss in activity (Davies et al., 1998) and this makes it suitable for high-heat processing such as spray drying. Due to the level of interest in nisin, many approaches for the production and purification of nisin have been developed, a selection of which are described in Table 2.1.

Nisin producing strains are commonly grown in standard growth media such as M17 (Abts et al., 2011), and MRS medium (Meghrous et al., 1997; Prioult et al., 2000), with glucose as the carbon source to produce nisin. In this study, *L. lactis* NZ9800 pLP712 (nisin A producing strain) was grown in a medium consisting of whey permeate supplemented with yeast extract as previously described (Bouksaim et al., 1998; Desjardins, Meghrous, & Lacroix, 2001; Xia, Chung, Yang, & Yousef, 2005). Whey permeate is primarily water, lactose and minerals and is what remains after the whey proteins have been harvested from whey (Song, Kim, Lee, & Hwang, 2007).

Due to differences in the methods of reporting results in the publications listed in Table 2.1, for example describing nisin purity in terms of total protein (Xiao et al., 2010) or in terms of total solids (Slootweg, Liskamp, & Rijkers, 2013), direct comparisons are difficult. However some clear trends are apparent. Purifications that used a commercial preparation as their starting material achieved greater yields and purity than those performed on the products of a nisin-producing culture when the same purification method was applied to both (Abts et al., 2011; Jozala et al., 2013). The highest purity was achieved by chromatographic and antibody-based approaches

(Abts et al., 2011; Jozala et al., 2015; Meghrous et al., 1997; Prioult et al., 2000) however, these approaches also gave the lowest yields. Non-chromatographic/antibody based approaches (Gonzalez-Toledo et al., 2010; Jozala et al., 2013; Kelly, Reuben, Rhoades, & Roller, 2000; Slootweg et al., 2013; Xiao et al., 2010), many of which were phase separation, achieved higher yields and would be easier to scale up, however they had a relatively lower purity. Of the published high yield approaches toluene (Kelly et al., 2000), dichloromethane (Slootweg et al., 2013) and ethanol (Xiao et al., 2010) gave the highest purity, however the use of toluene or dichloromethane is not ideal from a safety perspective.

Salting-out is a method commonly used for protein purification and works by salts drawing water molecules away from the hydrophobic regions in proteins and peptides. These hydrophobic regions then interact with each other, resulting in aggregates that precipitate out of solution (Scopes, 1994). However, pH, temperature, salt concentration and the concentration of the protein or peptide of interest in the solution all affect the effectiveness of the salting-out (Scopes, 1994).

While salting-out has been used in extracting nisin from culture broth, to the authors knowledge this has only been used as an initial stage of a longer purification process, with ammonium sulphate being the salt commonly used (Gujarathi, Bankar, & Ananthanarayan, 2008; Meghrous et al., 1997). In the method described here, the effectiveness of a simple salting-out procedure using sodium chloride to extract nisin from the cell-free supernatant (CFS) of a nisin producing culture and a commercial nisin preparation is outlined and compared to equivalent published methods.

Table 2.1. Published purification approaches for nisin.

Approach	Starting material	After purification				Reference
		Format	Scale of nisin component	Nisin content	Purification factor (on total protein basis)	
Ammonium sulphate precipitation followed by a series of reversed phase chromatographies culminating in reversed phase HPLC	Nisin producing culture	Solution	µg	Pure	~1600	(Bouksaim et al., 1998; Meghrous et al., 1997)
Ammonium sulphate precipitation followed by hydrophobic interaction and gel permeation chromatography	Nisin producing culture	Solution	n/a	n/a	~11	(Gujarathi et al., 2008)
Hydrophobic interaction chromatography	Commercial nisin preparation[a,b]	Solution	µg/mL	Pure	~270	(Jozala et al., 2015)
Hydrophobic interaction chromatography	Nisin producing culture	Solution	µg/mL	Pure	~770	(Jozala et al., 2015)
Cation exchange	Commercial nisin preparation[a,b]	Solution	mg	>98%	n/a	(Abts et al., 2011)
Cation exchange	Nisin producing culture	Solution	mg	n/a	n/a	(Abts et al., 2011)
Antibody-coated magnetic beads	Nisin producing culture	Solution	µg	63%[c]	~1100	(Prioult et al., 2000)
Expanded bed ion exchange chromatography	Nisin producing culture	Solution	mg	~4.6%[d]	31	(Cheigh et al., 2004)
Aqueous two-phase micellar system	Commercial nisin preparation[a,b]	Solution	mg/mL	~0.2%[e]	n/a	(Jozala et al., 2013)
Aqueous two-phase micellar system	Nisin producing culture	Solution	µg/mL	~0.1%[e]	n/a	(Jozala et al., 2013)
Ethanol extraction	Commercial nisin preparation[a,f]	Spray dried powder	n/a	~53%[c]	4.4	(Xiao et al., 2010)
Toluene extraction	Commercial nisin preparation[a,g]	Powder (by rotary evaporation)	mg	~51%[d]	n/a	(Kelly et al., 2000)
Dichloromethane extraction	Commercial nisin preparation[a,h]	Lyophilised powder	mg	34%[d]	n/a	(Slootweg et al., 2013)
Adsorption-desorption	Nisin producing culture	Lyophilised powder	mg	~0.3%[d]	n/a	(Gonzalez-Toledo et al., 2010)

[a] Powder is 2.5 % nisin (w/w) on a total solids basis
[b] From Sigma-Aldrich (St. Louis, Missouri, US)
[c] % w/w on a total protein basis
[d] % w/w on a total solids basis
[e] % w/w on a total mass basis
[f] From MP Biomedicals (Solon, Ohio, US)
[g] From DuPont (Beaminster, UK)
[h] From Chr. Hansen A/S (Hørsholm, Denmark)

2.3. Materials and methods

2.3.1. Production of nisin from a culture

For the preparation of the supplemented whey permeate (SWP) medium, dried whey permeate (WP, Kerry Group, Naas, Ireland) and yeast extract (YE, Merck Millipore, Darmstadt, Germany) were reconstituted in distilled water to a final concentration of 6% (w/v) WP and 2% (w/v) YE.

Fermentations were carried out in a FerMac 310/60 Bioreactor Fermenter (Electrolab Biotech, Tewkesbury, Gloucestershire, UK). The 5 L vessel was autoclaved twice at 121 °C for 15 min, with a one day waiting period between autoclaving to allow any surviving bacterial spores to germinate. The vessel was then filled with 4 L of SWP medium and autoclaved as described above.

L. lactis NZ9800 pLP712 which had been stocked in 20% glycerol (v/v) at -80 °C, was propagated twice in M17 (Terzaghi & Sandine, 1975) broth (Oxoid, Basingstoke, UK) supplemented with 0.5% lactose (VWR, Dublin, Ireland) (LM17), before 2 mL was used to inoculate 100 mL of SWP medium. The inoculum was grown overnight in 100 mL of SWP medium at 30 °C and the fermenter was then inoculated with 80 mL of the overnight culture.

The bioreactor was run at 30 °C and pH 6.0 (controlled by addition of 6 M NaOH) for 16 h. Mixing was performed by an impeller with four inclined flat blades at 2×*g*. The cells were removed by centrifugation at 11,270×*g* for 20 min at 4 °C and the CFS was collected.

2.3.2. Purification of nisin from a culture fermentate

Starting with 760 mL aliquots of the CFS, the pH was adjusted to 7 and the NaCl concentration was determined by conductivity. Each CFS aliquot was adjusted to a final NaCl concentration of 2.27 M, volume of 800 mL and pH of 7. This was centrifuged for 2 h at 4 °C and 16,900×*g*. The supernatant was discarded. For each 800 mL of volume before centrifugation, the pellet was suspended in water in a final volume of 30 mL. The pH of the suspended pellet was adjusted to 7. The suspended pellet was centrifuged for 2 h at 4 °C and 16,900×*g*. The supernatant was discarded and the pellet was collected.

Freeze-drying was performed on a Virtis Advantage (SP Scientific, Gardiner, New York, US), with the sample adjusted to a pH of 3 using HCl.

2.3.3. Purification of nisin from a commercial nisin preparation

Nisaplin® (DuPont, Beaminster, UK) (720 g) was suspended in water to a total volume of 2580 mL and centrifuged at 16,900×*g* for 15 min; the supernatant was discarded and the pellet was suspended in water to give a total volume of 860 mL. This was centrifuged at 16,900×*g* for 15 min, the supernatant was discarded and the pellet was suspended in 400 mL of water. The pH was adjusted to and maintained between 3 and 3.5 using HCl at all stages of the process.

The solution was spray dried on a B-191 spray dryer (Buchi, Flawil, Switzerland) using an inlet temperature of 180 °C and the flow rate adjusted to maintain an outlet temperature of 92 °C.

2.3.4. Cell counts

Viable cell counts were determined in culture samples by serially diluting the culture in maximum recovery diluent (MRD, Oxoid Ltd., Basingstoke, UK) and dilution plating on MRS agar (Oxoid Ltd., Basingstoke, UK) and LM17 agar (Merck Millipore, Darmstadt, Germany). The plates were incubated at 30 °C for 48 h and results expressed as colony forming units/mL (cfu/mL).

2.3.5. Lactose and lactic acid quantification

Lactose and lactic acid concentrations were determined using high performance liquid chromatography (HPLC) based on published methods (Desjardins et al., 2001; Xia et al., 2005). A Waters 2695 separation module with a Waters 2487 dual λ absorbance detector and a Waters 2414 refractive index detector, running on Waters Empower software (Waters, Dublin, Ireland) was used in conjunction with a Rezex™, RHM-Monosaccharide, 8% cross linked H+, 300 × 7.80 mm column from Phenomenex (Macclesfield, UK). The mobile phase, 0.0032 M H_2SO_4, was run at a flow rate of 0.5 mL/min and at 60 °C. Lactose was detected by refractive index and lactic acid by absorbance at 210 nm. The samples were quantified from the area of the peaks using standard curves.

2.3.6. Nisin quantification by reversed phase - high performance liquid chromatography (RP-HPLC)

The concentration of nisin was determined using RP-HPLC based on published methods (Buonocore et al., 2003; Chollet, Sebti, Martial-Gros, & Degraeve, 2008). RP-HPLC was carried out using a Waters e2695 separation module with a Waters 2489 UV/visible detector, running on Waters Empower software (Waters, Dublin, Ireland) and a reversed phase Jupiter, 5 µm, C18, 300 A, 250 mm × 4.6 mm column from Phenomenex (Macclesfield, UK). Solvent A was 0.1% (v/v) trifluoroacetic acid (TFA) (Sigma-Aldrich, Arklow, Ireland) in Milli-Q® water (Merck Millipore, Carrigtwohill, Ireland), and solvent B was 90% (v/v) HPLC-grade acetonitrile (Thermo Fisher Scientific, Dublin, Ireland) containing 0.1% TFA (v/v) in Milli-Q® water. A linear gradient from 22.2% B to 55.6% B over 30 min was run at a flow rate of 1.0 mL/min. Nisin was detected by absorbance at 214 nm and its peak corresponded to approximately 36% acetonitrile. Nisaplin® was used to generate a standard curve and the amount of nisin was calculated from the area of the peak at 214 nm. The concentration of nisin in Nisaplin® was 1.82% nisin (w/w) (DuPont, personal communication, 2016).

2.3.7. Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI TOF MS)

The molecular mass of the RP-HPLC fraction corresponding to the nisin peak was determined using MALDI TOF MS using an Axima TOF^2 (Shimadzu Biotech, Kyoto, Japan) as previously described (Field et al., 2012).

2.3.8. Nisin quantification by activity assay

The biological activity of nisin was estimated by agar diffusion activity assays (Ryan, Rea, Hill, & Ross, 1996). *L. lactis* subsp. *cremoris* HP (Lambie, Altermann, Leahy, & Kelly, 2014), the indicator strain, was grown overnight in M17 broth (Oxoid, Basingstoke, UK) containing 0.5% lactose (VWR, Dublin, Ireland) (LM17). LM17 agar was tempered to 45 °C and seeded with 0.5% of the indicator strain. Twenty millilitre aliquots of the seeded agar were dispensed into sterile petri dishes, these were allowed to solidify and wells of 5 mm in diameter were bored in the agar. Serial two-fold dilutions of the samples were dispensed into the wells in 50 µL aliquots and the plates were incubated overnight at 30 °C. The activity of the nisin resulted in zones of inhibition surrounding the wells. Nisin was quantified based on a published method (Bernbom et al., 2006) by plotting the area of the zone of inhibition against the log of the nisin concentration of a serial dilution of Nisaplin® to generate a linear standard curve. Nisin activity was also expressed as arbitrary units (AU) in terms of AU/mg (Ryan et al., 1996), which was calculated as the reciprocal of the lowest dilution that gave a definite zone of inhibition.

2.3.9. Conductivity

The concentration of NaCl in the sample was determined using a MultiLine® P3 conductivity meter (WTW, Weilheim, Germany) in conjunction with a NaCl standard curve.

2.3.10. Quantification of total protein

Total protein was quantified using the Kjeldahl method (ISO, 2004) using a Kjeltec™ 8400 Analyser in conjunction with a Kjeltec™ 8460 sampler (FOSS, Warrington, Cheshire, UK) with 6.25 used as the conversion factor.

2.4. Results and discussion

Two purification methods were developed to produce an enriched nisin powder using either a culture supernatant or a commercially available nisin preparation as the source of nisin (Fig. 2.1). In the case of the culture supernatant, nisin was produced by growing *L. lactis* NZ9800 pLP712 in a supplemented WP medium under controlled conditions of pH (6.0) and temperature (30 °C) for 16 h. At the end of the fermentation the viable cell count was 2.86×10^9 ($\pm$ 0.23×10^9) cfu/mL. Both purification methods involved using NaCl to precipitate the nisin peptides. In the case of the commercial preparation there was sufficient NaCl present in the Nisaplin® (~93% w/w) such that additional salt was not required to salt-out the nisin. As nisin is most stable at pH 3-3.5, it was preferable to perform the salting-out at this pH. The concentration of nisin in the commercial sample (Table 2.2) was sufficient for precipitation by salting-out at a pH of 3-3.5. The lower initial concentration of the nisin from the nisin producing culture (Table 2.2) required a higher pH (7.0) for efficient precipitation using the salting-out method. During the purification procedure the concentration of nisin was measured using RP-HPLC. The nisin peak in the RP-HPLC chromatograms (Fig. 2.2) was confirmed using MALDI-

TOF mass spectrometry, which showed a mass of 3354.09 Da (Fig. 2.2), correlating with the calculated mass of 3354.07 Da for nisin (Abts et al., 2011).

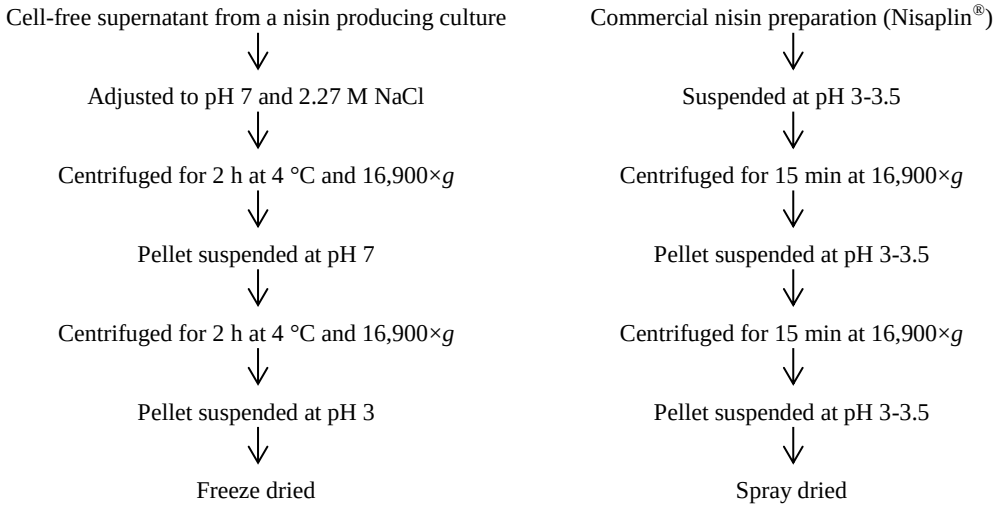

Fig 2.1. Flow chart of purification from the cell-free supernatant of a nisin producing culture (on left) and purification from a commercial preparation (on right).

Table 2.2. Purification of nisin using the salting-out approach from the CFS of a nisin producing culture (*L. lactis* NZ9800 pLP712) and a commercial preparation (Nisaplin®)

Purification of nisin from a nisin producing culture			Purification of nisin from a commercial preparation		
	Composition before purification (CFS)	Composition after purification and drying		Composition before purification (Nisaplin®)	Composition after purification and drying
Volume (mL)	760	n/a			
Total solids (mg)	62,500 (± 2,200)	64.2 (± 5.6)	Total solids (g)	720	10.9 (± 0.5)
Lactic acid (mg)	37,200 (± 450)	11.4 (± 0.5)			
NaCl (mg)	12,400 (± 130)	2.85 (± 0.30)	NaCl (g)	669 (± 6.0)	1.60 (± 0.08)
Total protein (mg)	8,350 (± 120)	46.7 (± 11.0)	Total protein (g)	27.7 (± 0.6)	7.83 (± 0.07)
Lactose (mg)	4,520 (± 110)	1.10 (± 0.14)			
Nisin (mg)	25.9 (± 0.7)	21.3 (± 4.7)	Nisin (g)	13.1	6.29 (± 0.44)

Mean ± SD, $n \geq 3$ except for lactose and lactic acid $n = 2$; the difference between before and after purification and drying are statistically significant ($p < 0.05$) for each component, except nisin from a nisin producing culture).

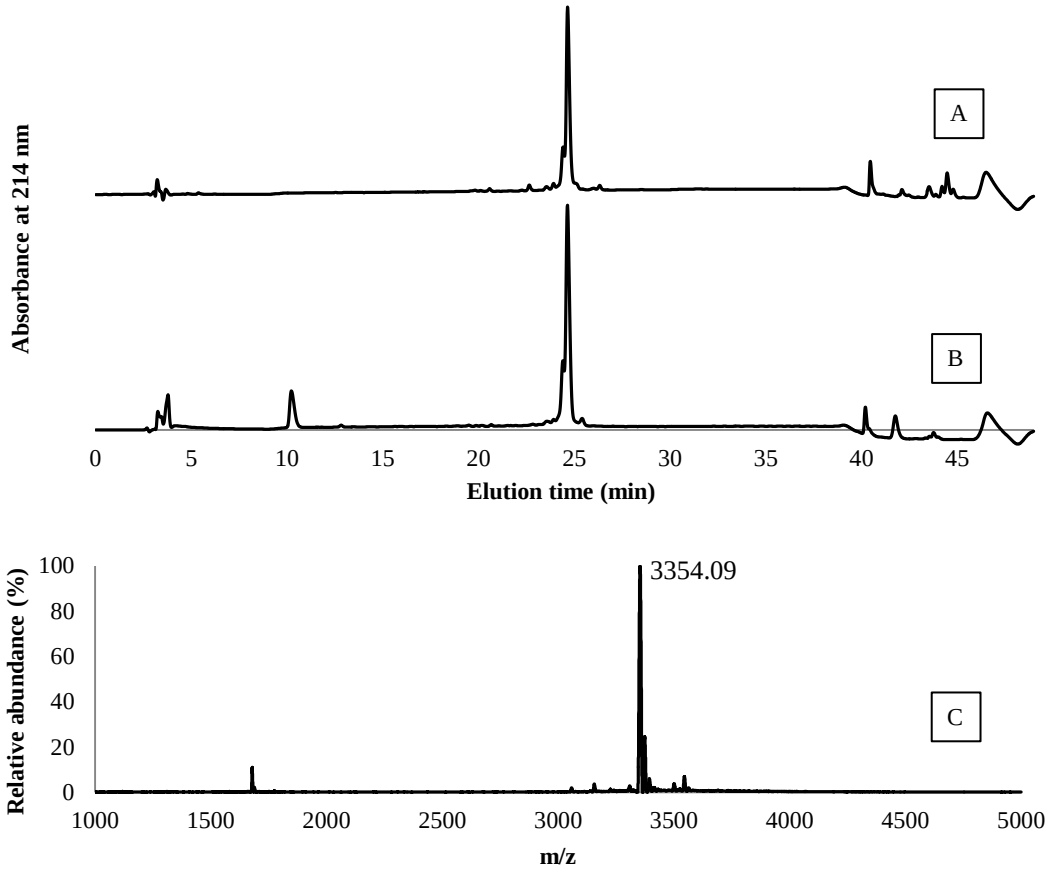

Fig. 2.2. RP-HPLC chromatograms of the purification products from a nisin producing culture (A) and a commercial nisin preparation (B). Nisin eluted at 24.7 min corresponding to approximately 36% acetonitrile. RP-HPLC nisin peak analysed by mass spectrometry (C). Molecular mass (3354.09 Da) correlates with that of nisin (3354.07 Da).

Table 2.3. Summary of the properties of the products of purification using the salting-out approach.

Approach	Starting material	After purification				
		Format	Scale of nisin component	Nisin content		Purification factor (on total protein basis)
Salting-out	Nisin producing culture	Lyophilised powder	mg	On total solids basis On total protein basis	33.3% 45.7%	148
Salting-out	Commercial nisin preparation[i]	Spray dried powder	g	On total solids basis On total protein basis	57.7% 80.3%	1.70

[i] From DuPont (Beaminster, UK)

Prior to purification, the major non-nisin components of the CFS, other than water, were lactic acid, NaCl, lactose, proteins and peptides. The purification process removed over 99% of each of these non-nisin components resulting in a powder containing approximately 33% nisin (Table 2.2). Using the same principle, a commercial nisin preparation was enriched from 1.82% nisin to approximately 58% nisin. The major non-nisin component in Nisaplin® is NaCl and this approach removes over 99% of the NaCl (Table 2.2). The nisin concentration of the product purified from Nisaplin® on a % w/w basis was 57.06 (± 0.51) when measured by RP-HPLC and 58.04 (± 0.05) when measured by the biological activity assay. The similarity of these results validates both assay methods. This also shows that nisin activity is not lost during processing. Table 2.3 presents the salting-out approach in the format used in Table 2.1 for the published purification approaches. When the activities of the purified products are expressed as AU/mg nisin, the purified product from a nisin producing culture had 1,296 (± 173, n = 3) AU/mg, whereas the purified product from a commercial preparation had 2,272 (± 812, n = 4) AU/mg.

As spray-drying is the most common method used to dry solutions or suspensions in the food industry (Jangam, 2011), it was used in the case of the commercial nisin powder; however due to the small volume of the nisin purified from a culture, this was freeze-dried (Fig. 2.1). Purification yield was calculated by expressing the amount of nisin in the purified product as a percentage of the total amount of nisin before purification and thus expressing how much of the original nisin was retained throughout the process. Purifying nisin from a culture gave a yield of 82%, whereas in the case of the commercial nisin powder the yield was 48% (Table 2.2). The lower yield for nisin purified from a commercial powder can be attributed to the spray drying stage as the losses at this stage represent 35% of the

original nisin. Laboratory scale spray drying is noted for its high sample losses (Maury et al., 2005; Soares e Silva et al., 2012), however larger spray dryers have much higher yields than laboratory scale dryers (Imtiaz-Ul Islam, Edrisi, & Langrish, 2013); for example, the industrial spray drying of milk is normally performed with yields in excess of 95% (Imtiaz-Ul Islam et al., 2013). Therefore it is likely the yields would be much greater if the process was scaled up.

2.5. Conclusion

The salting-out approach presented here produces powders containing 33 or 58% nisin, from a culture supernatant or a commercial nisin preparation, respectively. When compared to other high yield approaches that have equivalent starting materials, the salting-out approach gives higher purity; in addition it does not require organic solvents such as toluene or dichloromethane and the simplicity of the approach facilitates scale-up.

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Chapter 2 supplementary material (unpublished)

S2.1. Introduction

Due to the presence of a non nisin bacterial peptide (European Food Safety Authority, 2006) in the commercial nisin preparation (Nisaplin®), it was attempted to purify the nisin from the other protein components of the commercial nisin preparation using cation exchange fast protein liquid chromatography (FPLC).

S2.2. Materials and methods

The cation exchange FPLC procedure was developed based on Abts et al. (2011) with several modifications including using 8 times the column volume in order to upscale the procedure.

FPLC was carried out using an ÄKTA purifier and a XK26/20 column (40 mL column volume) containing a SP Sepharose® Fast Flow cation exchange chromatography resin (all FPLC components from GE Healthcare, Little Chalfont, UK). The flow rate was 5 mL/min and the peptides were detected by absorbance at 214 nm.

To prepare the sample for FPLC, 6.5 g of Nisaplin® was desalted overnight using benzoylated dialysis tubing (2,000 dalton molecular weight cut off, D7884, Sigma-Aldrich, Arklow, Ireland) in 50 mM acetic acid, pH 3.5. The desalted Nisaplin® solution had its total volume brought to 100 mL using 50 mM acetic acid pH 4.0 and was loaded onto the FPLC column. Unbound material was eluted with 6

column volumes of 200 mM NaCl, 50 mM acetic acid, pH 4.0. The nisin was eluted with 13 column volumes of 400 mM NaCl, 50 mM acetic acid, pH 4.0. Finally the column was washed with 6 column volumes of 50 mM acetic acid 1 M NaCl pH 4.0. The elution products were analysed by RP-HPLC which was performed as described in section 2.2.6. with the single modification that the linear gradient from 22.2% B to 55.6% was run over 15 min.

To remove the salt from the purified nisin in the 400 mM NaCl elute, it was precipitated with 20% (v/v) trichloroacetic acid (TCA) overnight at 4 °C. To remove the TCA, the purified nisin was washed twice with 4 °C acetone. To obtain the dry mass of the pellet it was freeze dried. The nisin content was quantified by RP-HPLC as described in section 2.2.6. The molecular mass of the non-nisin peptide was determined by MALDI TOF MS analysis of its RP-HPLC peak as described in section 2.2.7.

S2.3. Results

The non-nisin bacterial peptide was determined to have a molecular mass of 2351 Da. by MALDI TOF MS. In the chromatogram of Nisaplin® sample (Fig. S2.1A) the non-nisin bacterial peptide eluted at 11.4 min (26% acetonitrile) and the nisin eluted at 18.6 min (40.4% acetonitrile). The developed FPLC procedure successfully separated these 2 peptides with the non-nisin peptide eluting in 200 mM NaCl, 50 mM acetic acid, pH 4.0 and the nisin eluting in 400 mM NaCl, 50 mM acetic acid, pH 4.0 (Fig. S2.1).

The feed stock for this process was 6.5 g of Nisaplin® (118.3 mg of nisin) and the end produce after freeze drying had a mass of 83 mg, which was determined to contain 26.56 mg of nisin (32% nisin w/w) by RP-HPLC.

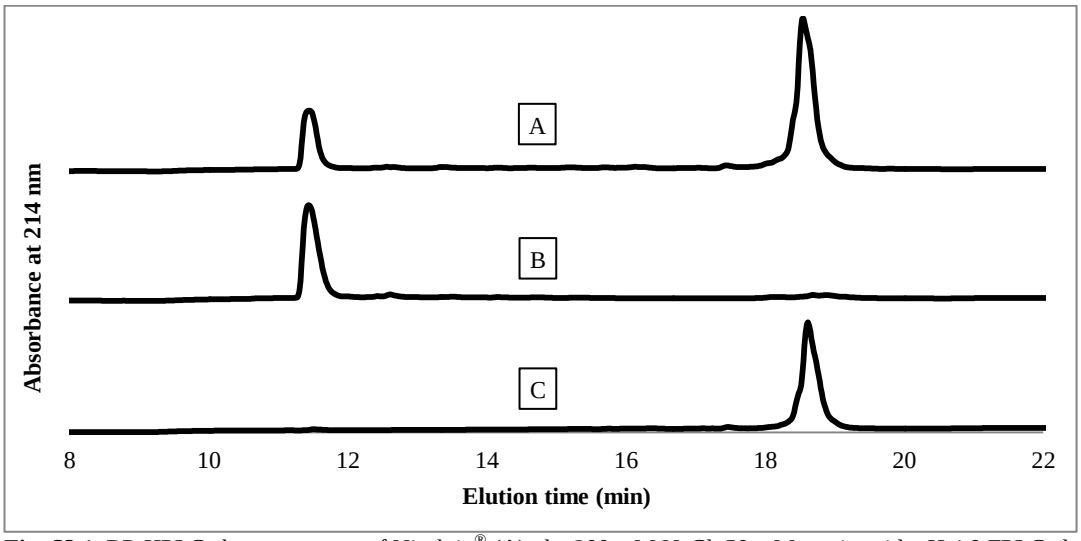

Fig. S2.1. RP-HPLC chromatograms of Nisalpin® (A), the 200 mM NaCl, 50 mM acetic acid, pH 4.0 FPLC elute (B) and the 400 mM NaCl, 50 mM acetic acid, pH 4.0 FPLC elute (C).

S2.4. Conclusion

Although this approach was successfully able to extract nisin from the non-nisin peptides in Nisaplin®, the yields were too low, particularly with regard to the process time required, for the procedure to be suitable for our purposes.

S2.5. References

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Chapter 3

Simulated gastrointestinal digestion of nisin and interaction between nisin and bile

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3.1. Abstract

Nisin, an antimicrobial peptide showing activity against many Gram positive bacteria, is widely used as a food preservative. The simulated gastrointestinal digestion of nisin (variant A) was studied using the *in vitro* INFOGEST digestion method. Following oral, gastric and small intestinal digestion, there was no intact nisin in the system and the nisin was primarily digested by pancreatin. After digestion, six nisin fragments (1-11, 1-12, 1-20, 1-21, 1-29 and 1-32) were identified by reversed phase high performance liquid chromatography and mass spectroscopy and four of these nisin fragments (1-20, 1-21, 1-29 and 1-32) demonstrated low antibacterial activity against *Lactococcus lactis* HP in agar diffusion activity assays. Additionally, it was observed that bile salts form a complex with nisin. This was examined by atomic force microscopy, turbidity and dynamic light scattering, which showed that this interaction resulted in significantly larger bile salt micelles. The presence of bile salts at physiological levels significantly altered the relative amounts of the nisin fragments 1-12, 1-20 and 1-29 produced during an *in vitro* digestion. This study highlights the importance of including bile in simulated digestions of antimicrobial peptides in order to obtain a more accurate simulation of the *in vivo* digestion products and their activity.

3.2. Introduction

Nisin is a 34 amino acid antimicrobial peptide produced by strains of *Lactococcus lactis* subsp. *lactis* that is active against many Gram-positive bacteria and is widely used as a food preservative (Gharsallaoui, Oulahal, Joly, & Degraeve,

2016). Nisin is extremely stable at pH 3 and can be autoclaved at this pH with <5% loss of activity (Davies et al., 1998), whereas above pH 6 it is unstable even at room temperature (Kelly, Reuben, Rhoades, & Roller, 2000).

The discovery that nisin is inactivated by pancreatin (Heinemann & Williams, 1966), primarily due to its chymotrypsin component (Jarvis & Mahoney, 1969), was a factor in nisin being awarded Generally Recognized As Safe (GRAS) status by the US Food and Drug Administration (FDA) (US Food and Drug Administration, 1988) and the European Food Safety Authority (EFSA) declaring that nisin is safe for use in food (European Food Safety Authority, 2006) with its assigned E number being E 234 (European Commission, 2011). It has been demonstrated more recently that nisin is also cleaved by the trypsin component of pancreatin (Chan et al., 1996). However these studies focused on pancreatic enzymes and did not take into account the other components of the digestive system such as bile.

Bile salts, the major functional component of bile, are biological surfactants which are involved in the digestion and absorption of lipids in the small intestine; in particular they transport the products of lipolysis in bile salt micelles to the sites of absorption (Bauer, Jakob, & Mosenthin, 2005). For the most common human and porcine bile salts, micelle formation takes place in two stages; hydrophobic interactions between bile salts results in primary micelles, which then interact via hydrogen bonding to form secondary micelles (Kandrac et al., 2006; Partay, Jedlovszky, & Sega, 2007; Small, 1968). The minimum bile salt concentration required for micelle formation is termed the critical micelle concentration (CMC). As the concentration of sodium ions affects the CMC, experiments with bile salts are commonly performed in 0.15 M Na^+ solutions to simulate physiological conditions

(Hofmann & Hagey, 2008). In a 0.15 M Na^+ solution, most bile salts have a CMC below 10 mM (Hofmann & Roda, 1984); 10 mM is also the bile salt concentration recommended for simulating physiological conditions during *in vitro* digestion (Minekus et al., 2014).

Previous digestion studies on nisin have focussed on pancreatic enzymes from the small intestine and those that investigated the nisin fragments produced by digestion used enzymes individually and often used digestions in excess of 20 h (Chan et al., 1996; Heinemann & Williams, 1966; Jarvis & Mahoney, 1969; Slootweg, Liskamp, & Rijkers, 2013). In order to study how nisin is digested under more physiologically relevant conditions, the INFOGEST method, a recently developed standardized static method for the digestion of food (Minekus et al., 2014) was utilised. This method is the consensus of an international network of scientists and is based on physiological conditions with each digestion comprising an oral, gastric and intestinal stage (Minekus et al., 2014). This approach would establish which nisin fragments are produced under physiological conditions and also their biological activity. In addition, by performing versions of the digestion without individual digestion components, the importance of non-proteolytic components such as bile on the digestion profile of nisin could be established.

3.3. Materials and methods

3.3.1. Materials

All reagents were obtained from Sigma-Aldrich (Arklow, Ireland) unless otherwise stated. For the simulated digestions the specific Sigma-Aldrich products

used were: salivary amylase (A1031), pepsin (P6887), bile (B8631) and pancreatin (P7545). Tween® 80 was obtained from Merck Millipore (Darmstadt, Germany). The nisin preparation used was Nisaplin® (DuPont, Beaminster, UK) (nisin variant A; referred to as 'nisin' throughout this text). This was enriched by salting out as previously described (Gough et al., 2017).

3.3.2. Simulated digestion

Simulated oral, gastric and small intestinal digestions were performed as described in the INFOGEST method (Minekus et al., 2014). Five variations of the digestion were performed: (i) nisin with all digestion components, (ii) nisin with all digestion components except bile, (iii) nisin with all digestion components except pancreatin, (iv) nisin with all digestion components except pepsin, bile and pancreatin, (v) all digestion components but no nisin. At least three replicates were performed for each of these five digestion setups. The initial nisin concentration was chosen so that the nisin concentration in the digestion product would be sufficient for quantification by reversed phase - high performance liquid chromatography (RP-HPLC). The digestion containing nisin and all digestion components was performed as follows: for the oral stage 5 mL of an 8.7 mg/mL nisin solution was combined with simulated salivary fluid (SSF) and salivary amylase (75 U/mL in final oral solution) to a final total volume of 10 mL; this was incubated at 37 °C for 2 minutes. For the gastric stage, the sample pH was adjusted to 3 using dilute HCl and combined with simulated gastric fluid (SGF) and pepsin (2,000 U/mL in final gastric solution) to a final total volume of 20 mL; this was incubated at 37 °C for 2 hours. For the small intestinal stage the pH was adjusted to 7 using dilute NaOH and

combined with simulated intestinal fluid (SIF) and bile (10 mM bile salts in final intestinal solution) and pancreatin (100 TAME U/mL in final intestinal volume) to a final total volume of 40 mL, this was incubated at 37 °C for 2 hours. The digestion products were snap-frozen in liquid nitrogen.

3.3.3. Determination of the effect of the presence of bile during digestion on the activity of the digestion products

To determine the effect of the presence of bile during digestion on the activity of the digestion products a simplified digestion method based on Minekus et al. (2014) was used; nisin was incubated with pancreatin in a MOPS buffer at pH 7 and 37 °C for 2 h with bile added either before or after digestion, with an equivalent volume of water added to samples that did not receive bile. The final constituents in each sample, in a total volume of 0.5 mL, were 100 µg/mL nisin, bile at a bile salt concentration of 0.3 mM, pancreatin at a concentration such that its trypsin activity was 100 TAME units per mL, 50 mM MOPS, 0.15 M NaCl and the pH was 7. The digestion products were analysed by activity assay as described in section 2.6.

3.3.4. Reversed phase - high performance liquid chromatography (RP-HPLC)

RP-HPLC was carried out on a Jupiter, 5 µm, C18, 300 Å, 250 mm × 4.6 mm column from Phenomenex (Macclesfield, UK) with an acetonitrile (Thermo Fisher Scientific, Dublin, Ireland) gradient as described previously (Gough et al., 2017). In

the case of digested nisin, fractions were collected throughout the gradient to determine the nisin fragments produced by digestion.

3.3.5. Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI TOF MS)

The molecular mass of the RP-HPLC peaks were determined using MALDI TOF MS using an Axima TOF^2 mass spectrometer (Shimadzu Biotech, Kyoto, Japan) as previously described (Field et al., 2012).

3.3.6. Activity assay

Biological activity was estimated by agar diffusion activity assays (Ryan, Rea, Hill, & Ross, 1996) in agar plates seeded with *Lactococcus lactis* subsp. *cremoris* HP which were prepared as described previously (Gough et al., 2017). Serial two-fold dilutions of the samples were performed in 0.15 M NaCl, 50 mM MOPS, pH 7. In specific cases a surfactant (0.3 mM bile salts, 8 mM Tween® 80 or 0.2 mM Triton™ X-100) was included in the diluent. The samples (50 μL) were dispensed into the wells and the plates incubated overnight at 30 °C. The activity of nisin resulted in zones of inhibition surrounding the wells. Activity is expressed as the minimum inhibitory concentration (MIC) in terms of μg/mL (Chan et al., 1996). MIC was calculated by plotting the area of the zone of inhibition at each dilution stage against the log of the nisin concentration (Bernbom et al., 2006); these had a linear relationship and the MIC was calculated from the equation of the line.

3.3.7. Atomic force microscopy (AFM)

For AFM, samples comprised 10 mM bile salts, 0.15 M NaCl, and 50 mM MOPS at pH 7, with or without 0.5 μg/mL nisin. The nisin concentration of 0.5 μg/mL was chosen as this is within the range that could occur in the small intestine after consumption of a nisin containing foodstuff (Delves-Broughton, 2005; Minekus et al., 2014). Aliquots (5 μL) were deposited onto freshly cleaved mica surfaces, dried in a desiccator and subsequently stored at ambient conditions to ensure equilibrated hydration. AFM images were obtained with an Asylum Research MFP-3D-AFM (Asylum Research UK Ltd., Oxford, UK) using AC-mode in ambient air. An aluminium reflex coated cantilever with a tetrahedral tip (AC 240), spring constant of 1.8 N/m (Olympus Optical Co. Ltd., Tokyo Japan), working frequency of 50-90 kHz, and scan rate of 0.5-1 Hz was used at a 512 × 512 resolution. The radius of curvature of the tetrahedral tip was 10 (± 3) nm.

3.3.8. Turbidity

Turbidity was measured at 600 nm as per (Dahmane, Lasia, & Zhao, 2008) using a Cary 100 Bio Spectrophotometer with temperature control (Agilent Technologies Ireland Ltd., Little Island, Ireland). The samples were prepared at pH 7 in a 50 mM MOPS buffer containing 0.15 M NaCl and analysed at 37 °C.

3.3.9. Dynamic light scattering

Z-average was measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). The samples were prepared in 0.15 M NaCl, adjusted to pH 7 using NaOH, filtered through a 0.22 µM PVDF filter (Gilson Scientific, Luton, UK) and analysed at 20 °C.

3.4. Results and discussion

3.4.1. Simulated digestion

Oral and gastric digestion of nisin without pepsin resulted in a 6% (± 0.6, n = 3) reduction in intact nisin when measured by RP-HPLC and the inclusion of pepsin brought the total reduction to 16% (± 2.2, n = 5); this limited digestion in the oral and gastric stages correlates with published results which show that nisin is primarily digested in the small intestine (Jarvis & Mahoney, 1969).

Gastrointestinal digestion without proteases or bile resulted in >50% loss in intact nisin (Table 3.1). As the oral and gastric stages caused limited reduction in nisin, this reduction can primarily be attributed to the small intestinal pH of 7 and temperature of 37 °C, as above pH 6 nisin is unstable with a temperature dependent decomposition rate (Kelly et al., 2000). It was noted that pH and temperature were not entirely responsible for the reduction in detectable nisin and that the simulated intestinal fluid, in particular its sodium bicarbonate component, played a minor role (data not shown).

Performing the small intestinal stage of digestion with bile and/or pancreatin resulted in no intact nisin being detectable by RP-HPLC (Table 3.1) and the products of digestions that included bile had greater antibacterial activity than similar digestions without bile.

Table 3.1. Products of *in vitro* gastrointestinal digestions of nisin.

Starting material	Oral, gastric and small intestinal digestion	Analysis of digestion products	
		% nisin (RP-HPLC)	MIC (or MIC equivalent)
Nisin solution	All components	0% (± 0)	22 µg/mL (± 5)
Nisin solution	All components except bile	0% (± 0)	41 µg/mL (± 2)
Nisin solution	All components except pancreatin	0% (± 0)	0.9 µg/mL (± 0.2)
Nisin solution	All components except pepsin, bile and pancreatin	48% (± 2)	2.9 µg/mL (± 0.5)
H_2O (no nisin)	All components	n/a	82 µg/mL (± 21)
Nisin solution	Not digested	100%	1.8 µg/mL (± 0.1)

The digestion products were analysed by RP-HPLC and agar diffusion activity assay. The amount of nisin detected by RP-HPLC is expressed as a % of the total initial nisin. Activity is expressed as the minimum inhibitory concentration (MIC, µg/mL). An equivalent MIC is given for the products of digestions without nisin; SD in brackets, $n \geq 3$.

The highest antibacterial activity was in digestions without pancreatin; this correlates with previous reports that pancreatin is primarily responsible for nisin digestion (Heinemann & Williams, 1966). However in digestions with all components except pancreatin, there was no intact nisin detected by RP-HPLC. The high antibacterial activity implies that intact nisin was present and suggests another digestion component may be affecting the behaviour of nisin on the RP-HPLC column, thus interfering with its detection. This component appeared to be bile, as digestions without bile or proteases had detectable nisin in their products.

3.4.2. Analysis of nisin fragments

As the products of digestions involving pancreatin demonstrated antibacterial activity that could not be accounted for by the bile or digestive enzymes, the activity

was likely due to fragments of nisin. To determine which nisin fragments were produced and which of these were bioactive, the digestion products were separated by RP-HPLC and the fractions collected (Fig. 3.1B). The fractions were analysed using MALDI-TOF mass spectrometry and by activity assay (Fig. 3.2). Digestions with pancreatin produced peptides with molecular masses corresponding to the theoretical and published molecular masses of nisin fragments 1-12, 1-20, 1-21, 1-29 and 1-32 and also a peptide with a molecular mass within two daltons of the theoretical mass of nisin fragment 1-11 (Table 3.2).

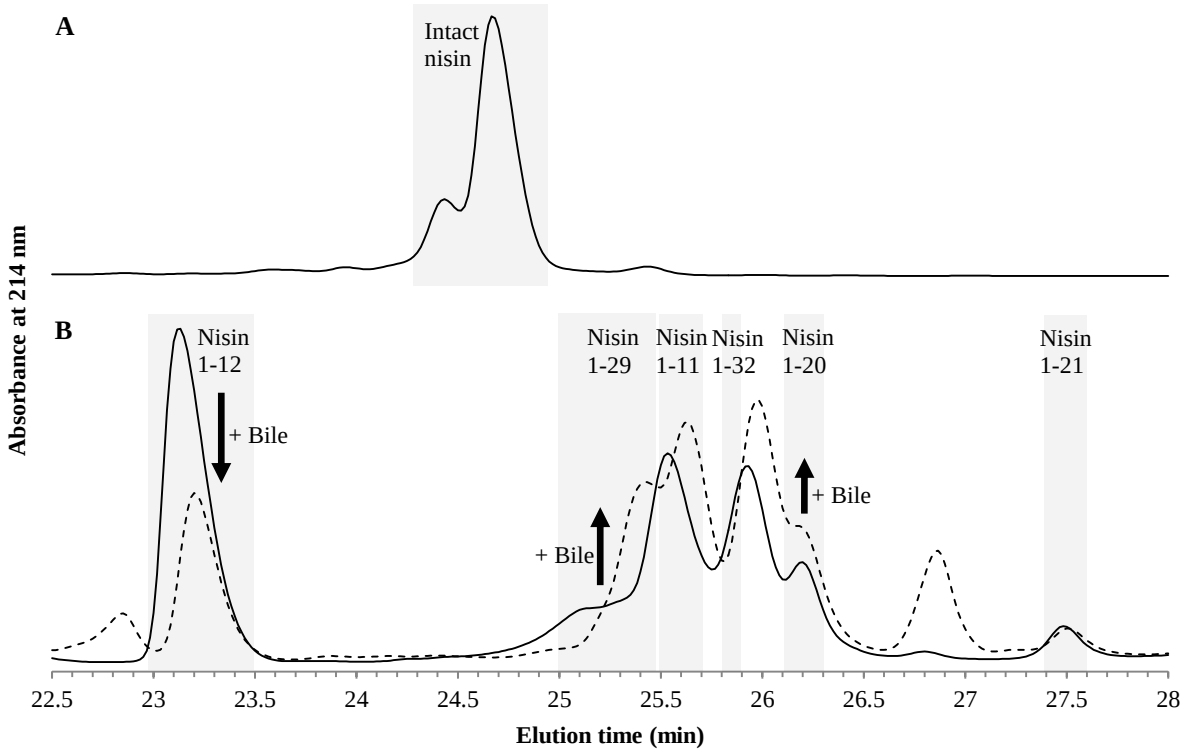

Fig. 3.1. RP-HPLC chromatograms of (A) undigested nisin and (B) the products of nisin digestion without bile (—) and with bile included (----). Regions where nisin fragments and intact nisin eluted are highlighted. The effect of including or excluding bile from a digestion with respect to the amount of nisin fragments 1-12, 1-29 and 1-20 produced is highlighted.

Table 3.2. Nisin fragments detected in the products of digestion.

Nisin fragment	Observed / predicted molecular mass (Da)	Antibacterial activity	Effect of inclusion of bile in digestion on height of corresponding peak in RP-HPLC chromatogram
1-11	1023 / 1021	None detected	Peak height not determinable due to background interference from co-eluting bile and pancreatin
1-12	1151 / 1150[b]	None detected	1.9 (± 0.3) fold decrease ($p = 0.0009$)
1-20	1881 / 1881[a]	Yes	1.4 (± 0.1) fold increase ($p = 0.02$)
1-21	2013 / 2012[b]	Yes	Not significant ($p = 0.06$)
1-29	2810 / 2809[a]	Yes	3.5 (± 0.3) fold increase ($p < 0.0001$)
1-32	3159 / 3157[a]	Yes	Peak height not determinable due to background interference from co-eluting pancreatin

Mean fold increases and SD are derived from three sets of replicates; p values are in brackets.
[a]Chan et al. (1996).
[b]Slootweg et al. (2013).

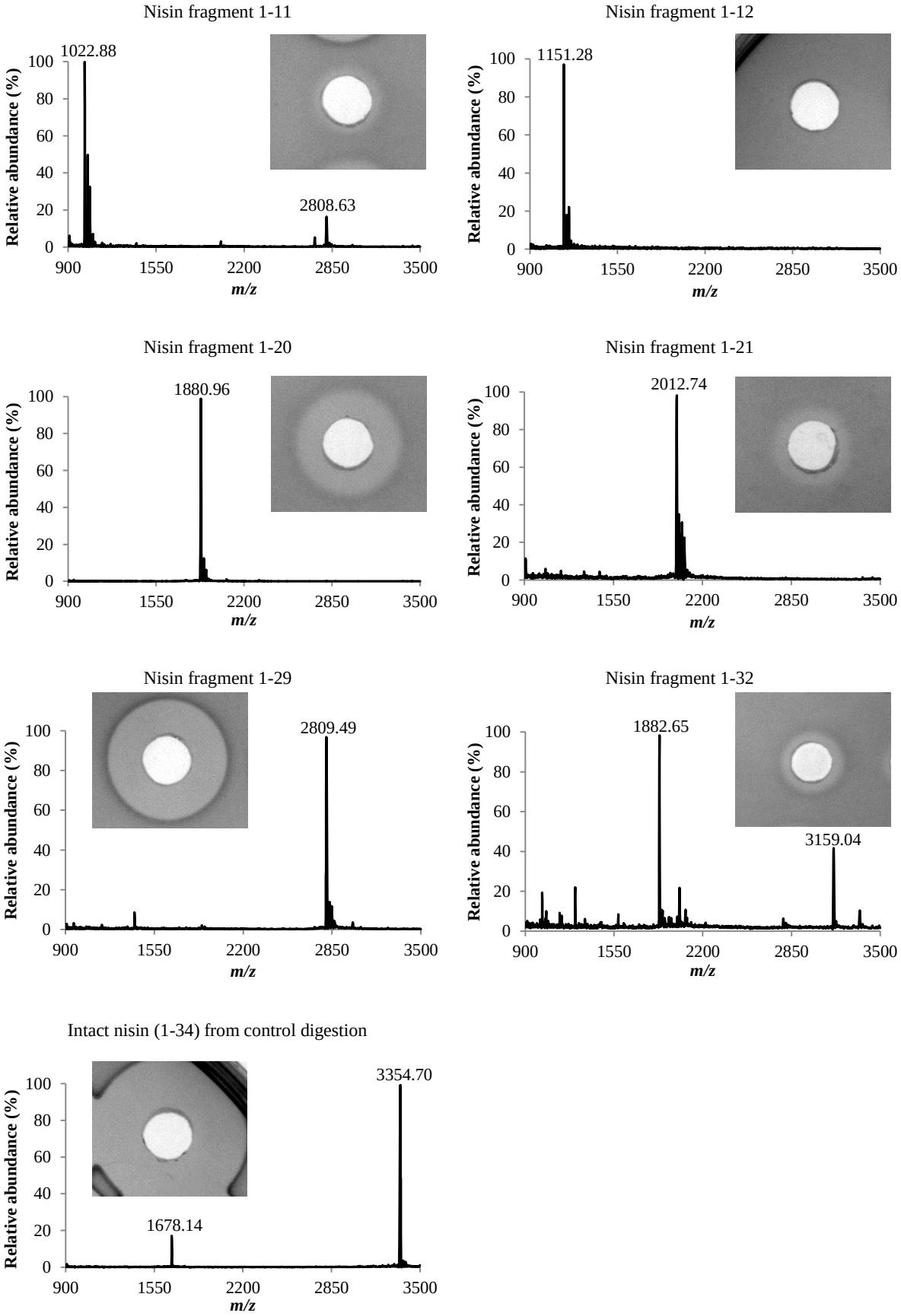

Fig. 3.2. Mass spectrometry analysis and agar diffusion activity assay (inserts) of nisin fragments produced by the digestion of nisin. Analysis of intact nisin from the products of the control digestion is included for comparison in which 3354.70 Da correlates with the predicted molecular mass of intact nisin (3355.12 Da) (Chan et al., 1996) and 1678.14 Da correlates with the molecular mass of doubly charged intact nisin.

Major peaks on the RP-HPLC traces (Fig. 3.1A and B) corresponded to nisin 1-12, 1-20, 1-29 and intact nisin, whereas nisin 1-21 gave a minor peak. Intact nisin had a shoulder region corresponding to the nisin variant [Ser^{33}]-nisin in which the serine residue at position 33 did not undergo post-translational modification to dehydroalanine (Chan et al., 1996). The peaks corresponding to nisin fragments 1-11 and nisin 1-32 were obscured by the co-eluting bile and pancreatic components. Although the RP-HPLC peak of nisin 1-32 was completely obscured by the background, its elution point was identified by activity assay.

Nisin 1-29 eluted at slightly different time points depending on whether the digestion was performed without bile (25.1 min) or with bile included (25.4 min). The peaks at 22.8 min and 26.9 min and the four major peaks between 25.5 min and 26.1 min were primarily due to bile and pancreatin and occurred in the control digestions that did not have nisin. The presence of bile in the digestion affected the peak heights of nisin fragments 1-12, 1-20 and 1-29 (Fig. 3.1B).

RP-HPLC fractionation did not lead to pure peptide fractions due to overlap between the elution of the fragments, for example in Fig. 3.2 nisin fragment 1-29 was detected in the mass spectrometry analysis of the elution peak of nisin fragment 1-11 and was most likely the source of the antimicrobial activity in the activity assay of nisin fragment 1-11, also nisin fragment 1-20 was visible in the mass spectrometry analysis of the elution peak of nisin fragment 1-32.

Nisin fragment 1-32 was not detected in the products of digestion in the presence of bile; as bile interfered with the detection of intact nisin (1-34) by RP-HPLC, it is proposed this also occurs with nisin fragment 1-32.

Nisin fragments 1-12, 1-20 and 1-21 have previously been produced by digests with trypsin or chymotrypsin for a minimum of 16 hours (Chan et al., 1996; Slootweg et al., 2013). Nisin fragment 1-29 has been produced by an 8 hour digestion with the bacterial protease thermolysin and by a 4 hour digestion of the nisin variant ([Ser^{33}]-nisin) with carboxypeptidase Y (Chan et al., 1996). Nisin fragment 1-32 has been produced by a 6 day acid treatment (Chan, Bycroft, Lian, & Roberts, 1989; Chan et al., 1996). To the authors knowledge it has not been previously demonstrated that these fragments can be produced under physiological conditions.

In Table 3.2 it is shown that the molecular masses observed in Fig. 3.2 are within two daltons of the predicted masses and that the inclusion of bile in a digestion altered the proportions of the nisin fragments produced when compared by peak height in a RP-HPLC chromatogram. In this study all the nisin fragments corresponding to amino acids 1-20 or longer demonstrated antibacterial activity against *Lactococcus lactis* (Fig. 3.2), which is in agreement with Chan et al. (1996). The decrease in nisin 1-12 and an increase in nisin 1-20 and 1-29, which are observed in Fig. 3.1B are shown to be significant (Table 3.2). This implies that the bile reduces the cleavage of nisin during digestion.

3.4.3. Nisin interaction with bile and other surfactants

As bile had been shown to increase nisin activity (Table 3.1) and alter its digestion (Table 3.2) the bile-nisin interaction was further examined and compared to nisin's interaction with other surfactants. The surfactants Tween® 80 and Triton™ X-114 were chosen for comparison as they increase the activity of nisin (Joosten &

Nunez, 1995; Jozala et al., 2008). Triton™ X-100 was substituted for Triton™ X-114, as Triton™ X-114 phase separates at the incubation temperature of the activity assay (30 °C) (Bordier, 1981). Regarding the concentrations used; 10 mM bile salts is physiological concentration (Minekus et al., 2014), while 0.3 mM bile salts and 0.2 mM Triton™ X-100 were the highest concentrations that did not cause antibacterial activity in activity assays. While Tween® 80 did not have an antibacterial affect at concentrations >8 mM, 8 mM was chosen because higher concentrations had a noticeable effect on viscosity. All these surfactants were at a molar excess over the nisin component (100 μg/mL nisin ≈ 0.03 mM nisin).

To investigate how surfactants affected the MIC of nisin when determined by activity assays; serial dilutions were performed in a MOPS/NaCl diluent on its own and with each of the surfactants, with a starting nisin concentration of 100 μg/mL (Table 3.3). Bile caused a reduction in MIC compared to the control, however a greater reduction was caused by Tween® 80 and Triton™ X-100 and both of these reduced the MIC by the same amount (Table 3.3). This implies that nisin interacts differently with bile compared to the other surfactants.

Table 3.3. Effect of surfactants on the minimum inhibitory concentration (MIC, μg/mL) of nisin in agar diffusion activity assays (SD in brackets, n = 3).

Surfactant	MIC (μg/mL)
No surfactant	1.81 (± 0.11)
0.3 mM bile salts	0.05 (± 0.01)
8 mM Tween® 80	0.008 (± 0.001)
0.2 mM Triton™ X-100	0.008 (± 0.001)

Surfactants can increase the activity of bioactive peptides in activity assays by reducing or preventing binding to glass or polypropylene assay containers through competition with the peptides for binding sites on the container or interacting with the binding sites on the peptides, primarily through hydrophobic

interactions (Duncan, Lee, & Warchol, 1995; Joosten & Nunez, 1995). If nisin activity was increased by bile, through the formation of a peptide-surfactant complex, there would also be an increase in the particle size of the bile salt micelle.

The effect of nisin-surfactant interaction on particle size was examined by AFM, turbidity and DLS. AFM analysis of bile with and without nisin (Fig. 3.3A and B) showed individual particles whose cross-sections had z-heights ranging from 20 to 190 nm, which was similar to the z-average means obtained by DLS for bile (100 nm ± 5) and bile with nisin (118 nm ± 9) (Fig. 3.3D) and similar to the published values for bile salt secondary micelles (50 to 200 nm) (Hildebrand, Garidel, Neubert, & Blume, 2004).

The turbidity of a system relates to both the size and density of particles that scatter light. In Fig. 3.3C, nisin was solubilised in a range of surfactants and turbidity increased with increasing nisin concentration, with the greatest turbidity increases occurring in the presence of bile. DLS (Fig. 3.3D) found that the z-average diameter of 10 mM bile salts with 100 μg/mL nisin was significantly larger than that without nisin ($p = 0.0123$, $n = 4$). There was no significant change in particle size when nisin was in solution with Triton™ X-100 or Tween® 80 (data not shown). To produce results of suitable quality by DLS, the samples were filtered and measured at 20 °C and pH 7 without a buffer.

The particle size analysis (Fig. 3.3) suggests that nisin formed a complex with bile and this was different to its interaction with other surfactants. In the activity analysis (Table 3.3) bile caused less of an increase in nisin activity than the other surfactants; this could be explained by bile forming a complex with nisin whereas the other surfactants bound to the assay container and thus reduced non-specific nisin binding in a different way. In the products of digestions which contained all

digestion components except pancreatin (Table 3.1), nisin was detected by activity assay but not detected by RP-HPLC; nisin could be favouring hydrophobic interaction with bile over hydrophobic interactions with the RP-HPLC column.

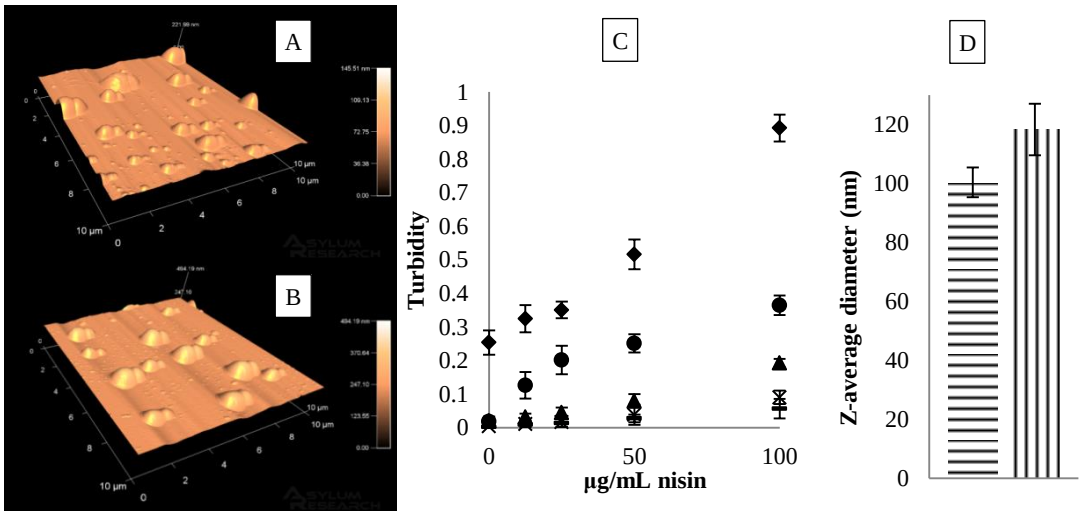

Fig. 3.3. Effect of nisin-surfactant interaction on particle size as examined by atomic force microscopy (AFM) (A and B), turbidity (C) and dynamic light scattering (DLS) (D). AFM: AFM of bile (A) and AFM of bile with nisin (B). Turbidity (C): ◆ 10 mM bile salts, ● 0.3 mM bile salts, ▲ 0.2 mM Triton™ X-100, ✕ 8 mM Tween® 80 and ▬ No surfactant (± SD, n = 4). DLS (D): ≡ 10 mM bile salts and ||| 10 mM bile salts with 100 µg/mL nisin (± SD, n = 4). Difference in particle size determined by DLS (D) is significant (p = 0.0123, n = 4).

With respect to the mechanism for bile-nisin interaction, both hydrophobic and ionic interactions are possible. Bile salts are anionic with a negative charge on an amino acid that is attached to one end of the main body of the bile salt via a short hydrocarbon chain (Fig. 3.4) (Hofmann & Hagey, 2008; Small, 1968). Nisin has a pI of 8.5 and thus is cationic under physiological conditions with the bulk of the positive charge being in the C-terminal domain (Fig. 3.4) and this is responsible for its initial interaction with its negatively charged targets (Breukink et al., 1997). The oppositely charged terminal regions of bile salts and nisin make ionic interactions likely. Bile salts are planar amphipathic molecules and thus have a hydrophobic and a hydrophilic side (Fig. 3.4); when forming a primary micelle they orientate their hydrophobic sides towards each other, giving the micelle a hydrophobic core (Fig.

3.4) (Hofmann & Hagey, 2008). Nisin is also amphipathic with the C-terminal being hydrophilic while the N-terminal is hydrophobic (Fig. 3.4) (Gharsallaoui et al., 2016). The hydrophobic N-terminal region of nisin has been reported to interact with the hydrophobic cores of surfactants such as dodecylphosphocholine and sodium dodecyl sulphate micelles (van den Hooven et al., 1996). It is therefore possible that the hydrophobic N-terminal region of nisin also interacts with the hydrophobic core of bile salt micelles. The reduced cleavage in the N-terminal region of nisin when a simulated gastrointestinal digestion is performed in the presence of bile (Table 3.2) may be due to the bile salts interacting with and surrounding the N-terminal region of the nisin and limiting the capacity of proteolytic enzymes to interact with the N-terminal region.

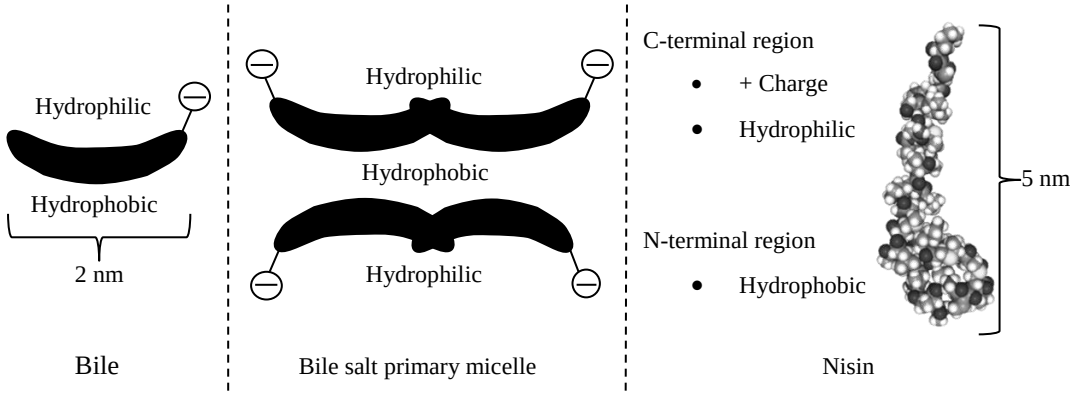

Fig. 3.4. Schematics of a bile salt, a bile salt primary micelle and a nisin peptide. A bile salt molecule is ~2 nm long (Small, 1971) and a nisin peptide is ~5 nm long (Sahl, 1994). Drawings of a bile salt and primary micelle are based on those of Small (1968). Bile salt primary micelles can consist of 2 to 10 bile salts (Li et al., 2009). The image of nisin was produced using Protein Database entry 1WCO (Hsu et al., 2004) in conjunction with the NGL Viewer (Rose & Hildebrand, 2015).

Having shown that nisin formed a complex with bile salts, which altered its digestion products, it was investigated how much this affected its antibacterial activity after digestion. The loss of nisin activity during digestion is primarily due to pancreatic enzymes (Section 3.1). To look specifically at whether bile could attenuate the loss of nisin activity due to pancreatin, a simplified digestion was

performed with pancreatin in buffer and bile added at the beginning or end of the digestion and the activity of the product determined by activity assay. The final concentration of bile salts was 0.3 mM so that the antibacterial activity of bile salts would not distort the results of the activity assay. The MOPS/NaCl buffer (pH 7) that was used as a diluent for the activity assay contained 0.3 mM bile salts, so that the surfactant effect would be consistent at all stages of the assay. The addition of bile before or after a 2 h digestion resulted in MICs of 11.8 μg/mL (± 0.3, n = 3) and 12.7 μg/mL (± 0.2, n = 3) respectively. Although statistically significant ($p = 0.01$), the difference in activity was slight.

Although the presences or absence of bile in a static *in vitro* digestion had a significant effect on the nisin fragments produced, this in turn had a negligible effect on antibacterial activity. As nisin fragments 1-12, 1-20 and 1-29 have low antibacterial activity (<6% the activity of intact nisin against *L. lactis* (Chan et al., 1996)) it is assumed that increases or decreases in their amount had a minimal effect on overall antibacterial activity.

3.5. Conclusions

Nisin was digested by intestinal proteases as has been previously highlighted (Heinemann & Williams, 1966; Jarvis & Mahoney, 1969) although intestinal pH and temperature by themselves also caused significant loss in nisin. The digestion products include six nisin fragments, four of which have limited antibacterial activity. Although nisin fragments have been previously identified (Chan et al., 1996; Slootweg et al., 2013), this is the first time that fragments generated during an *in vitro* digestion under physiological conditions have been identified. Nisin interacts

with bile, forming a complex that alters the relative amounts of the nisin fragments produced by digestion. This study highlights the importance of including bile in simulated digestions of antimicrobial peptides regardless of the presence or absence of a lipid component in the test samples.

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Chapter 4

Entrapment of nisin in starch for colonic delivery using spray coating and co-spray drying

4.1. Abstract

Bioactive proteins and peptides have numerous health benefits; however they can be digested during gastrointestinal transit if taken orally. Entrapment is an established method for oral delivery of bioactives, particularly to the colon; however the majority of current approaches come from a pharmaceutical direction and thus have aspects that would be less than ideal for a food product such as relatively large particles and ingredients that are not clean-label. A potential clean-label entrapment material is resistant starch, which is the portion of starch that resists digestion in the upper gastrointestinal tract but can be digested by bacteria in the colon; high amylose corn starch (HACS) is particularly high in resistant starch. Therefore HACS entrapment systems based on producing particles <100 μm in diameter were investigated. As a model bioactive peptide, the well characterised antimicrobial peptide nisin was used. Two approaches were used to apply this HACS coating, spray coating and co-spray drying. As spray coating is normally performed with particles >100 μm in diameter, particularly tablets, a carrier was needed to achieve suitable flowability for coating and a whey protein isolate (WPI) was found to be suitable. The nisin was successfully entrapped in HACS by spray coating; however this HACS coating was not resistant to treatment with pancreatic α-amylase at physiological concentration. Co-spray drying of nisin and HACS resulted in solid gels that had the capacity to resist digestion; however the nisin was not bound by the HACS gel. Although nisin was successively entrapped in HACS using both approaches, neither would be suitable for delivering nisin to the colon.

4.2. Introduction

Bioactive proteins and peptides have numerous health benefits, such as anticarcinogenic and antimicrobial properties, however if taken orally they can be inactivated during gastrointestinal transit, particularly due to the low pH and pepsin in the stomach, and the trypsin, chymotrypsin and carboxypeptidase in the small intestine (Goodman, 2010; Segura-Campos, Chel-Guerrero, Betancur-Ancona, & Hernandez-Escalante, 2011).

Although this makes delivering peptides to the colon particularly challenging, lower levels of proteases and higher responsiveness to permeation enhancers has made it a site of interest for the delivery of peptides such as insulin and calcitonin (Chen et al., 2017; Fetih et al., 2006; Maroni et al., 2012; Petersen et al., 2013). Additionally colonic delivery is essential for bioactive peptides that have a local effect in the colon, particularly antibacterial peptides that can be used to modulate the colonic microbiota.

Antimicrobial peptides (AMPs) are continually gaining interest as an alternative to antibiotics due to the rise in antibiotic resistance; in particular the bacteriocins, which are bacterially produced and do not exhibit significant toxicity towards mammalian cells unlike other AMP classes (Allen, Trachsel, Looft, & Casey, 2014). The narrow spectrum of the majority of these bacteriocins allows treatment of gastrointestinal infections without disrupting the native bacteria (Cotter, Ross, & Hill, 2013). An example of which is thuricin CD, which has a narrow spectrum of activity against the antibiotic resistant bacteria *Clostridium difficile* (Rea et al., 2010), however like most bioactive peptides, it is digested during gastrointestinal transit when taken orally (Rea et al., 2014).

Nisin is a bioactive peptide that has activity against a wide range of Gram positive bacteria and is widely used as a food preservative (Abee & Delves-Broughton, 2003). When taken orally, nisin is digested during gastrointestinal transit (Younes et al., 2017). Due to its commercial availability and thorough characterisation, nisin is considered suitable as a model bioactive peptide for colonic delivery systems (Habib & Sakr, 1999; Mallen, 2017; Ugurlu, Turkoglu, Gurer, & Akarsu, 2007).

As described in Chapter 1, the primary approaches for protecting a peptide during gastric transit to the colon are protease inhibitors, structural modification of the peptide and encapsulating the peptide in a protective coating. Protease inhibitors interfere with normal nutrient absorption and are carcinogenic with frequent use (Bernkop-Schnurch, 1998), whereas structural modification would require a specific system for each peptide to ensure the modifications that did not interfere with the activity of the peptide, making these options less than ideal.

As described in Chapter 1, release from a protective coating for colonic delivery can be based on pH, time or digestion by colonic bacteria. Due to the relative pHs of the small and large intestine, pH based release in the colon is not possible and colon targeted pH release systems normally begin releasing in the ileum of the small intestine (McConnell, Short, & Basit, 2008) whereas time based system are based on a controlled continual release, which is normally controlled by adjusting the rate of swelling (Del Curto et al., 2014; Maroni et al., 2016; Yuan, Jacquier, & O'Riordan, 2018). Protective coatings that are designed to be digested by colonic bacteria are generally based on carbohydrate polymers such as chitosan, pectin and starch (and its components such as amylose). These polymers are normally used in conjunction with a binder such as ethyl cellulose (EC) or

hydroxypropyl methylcellulose (HPMC) or a crosslinking agent such glutaraldehyde (Shukla & Tiwari, 2012; Sinha & Kumria, 2001).

Starch which is comprised of the carbohydrate polymers amylose and amylopectin, is of particular interest as a protective coating material as it can self-crosslink; by heating and cooling starch in the presence of water it can form solid gels, this process is known as gelatinisation and retrogradation (Liu, 2005). Resistant starch, is starch that is resistant to digestion in the upper gastrointestinal tract, but can be digested by bacteria in the colon, making it suitable for use as a protective coating that could provide colonic delivery (Basit, 2005). The portion of starch that resists digestion varies between starch source and type; high amylose corn starch (HACS, 70% amylose starch from maize) contains 46% resistant starch on a w/w basis (McCleary, McNally, & Rossiter, 2002).

When starch (or its component polymers) are used as a protective coating for colonic delivery, they are normally combined with a binder/plasticizer to control swelling in aqueous solution and increase structural integrity such as ethyl cellulose (Freire et al., 2010; McConnell et al., 2007; Wilson & Basit, 2005), triacetin (Pu et al., 2011) and acrylate polymers (Milojevic et al., 1996).

Two of the most common techniques for the entrapment of bioactives in a protective coating are co-spray drying and fluidised bed spray coating (de Vos, Faas, Spasojevic, & Sikkema, 2010), with the Wurster process being one of the most common for spray coating (Asija, 2012; Saleh & Guigon, 2007). Spray coating is normally performed with cores of diameter significantly greater than >0.1 mm, as smaller cores have a tendency to agglomerate and are difficult to fluidize (Gupta & Sathiyamoorthy, 1998; To & Dave, 2016).

Co-spray drying and spray coating have been used in many colonic delivery systems based on starch and its component polymers components (Desai, 2007; Desai, 2005; Dimantov, Greenberg, Kesselman, & Shimoni, 2004; Freire et al., 2010; Krogars et al., 2003; McConnell et al., 2007; Milojevic et al., 1996; Palviainen et al., 2001; Pu et al., 2011; Wilson & Basit, 2005). Very few of these studies (McConnell et al., 2007; Milojevic et al., 1996; Palviainen et al., 2001) applied the starch/amylose such that it would retrograded (crosslinking) into a solid gel during coating, this is likely due to the technical challenge of such an approach.

The cores produced in studies involving spray coating with starch/amylose have ranged in diameter from 0.3 to 8 mm (Pu et al., 2011; Wilson & Basit, 2005), however in the majority of studies where the core diameter was stated, this diameter was ≥1 mm (Freire et al., 2010; McConnell et al., 2007; Milojevic et al., 1996; Wilson & Basit, 2005).

In this study, nisin was entrapped in HACS using Wurster spray coating and co-spray drying, to protect it from digestion during gastrointestinal transit and enable it to be delivered to the colon. Spray coating was performed in two stages; firstly the nisin was combined with a carrier to produce a core and then the core was coated with HACS, whereas in co-spray drying the nisin and HACS were combined directly (Fig. 4.1).

There were two major differences with previous starch/amylose based approaches to create a product more suitable for inclusion in foodstuffs: the coatings had a clean-label composition (Asioli et al., 2017) comprising of only water and starch and the particles were of a smaller size, with the cores for spray coating <0.1 mm.

As mentioned earlier, small particles have poor flowability in spray coating, so in order to achieve suitable flowability in the spray coater with a <0.1 mm core, a carrier was required. Lactose is well established as carrier (Hamishehkar, Rahimpour, & Javadzadeh, 2012) and maltodextrin and trehalose are also used as carriers (Adler & Lee, 1999; Shrestha et al., 2007; Wang & Zhou, 2012) and thus they were investigated first. In order to achieve a cohesive coating using only water and starch it was necessary that the coating was applied in gelatinised state so that it could retrograde into a solid gel *in situ*.

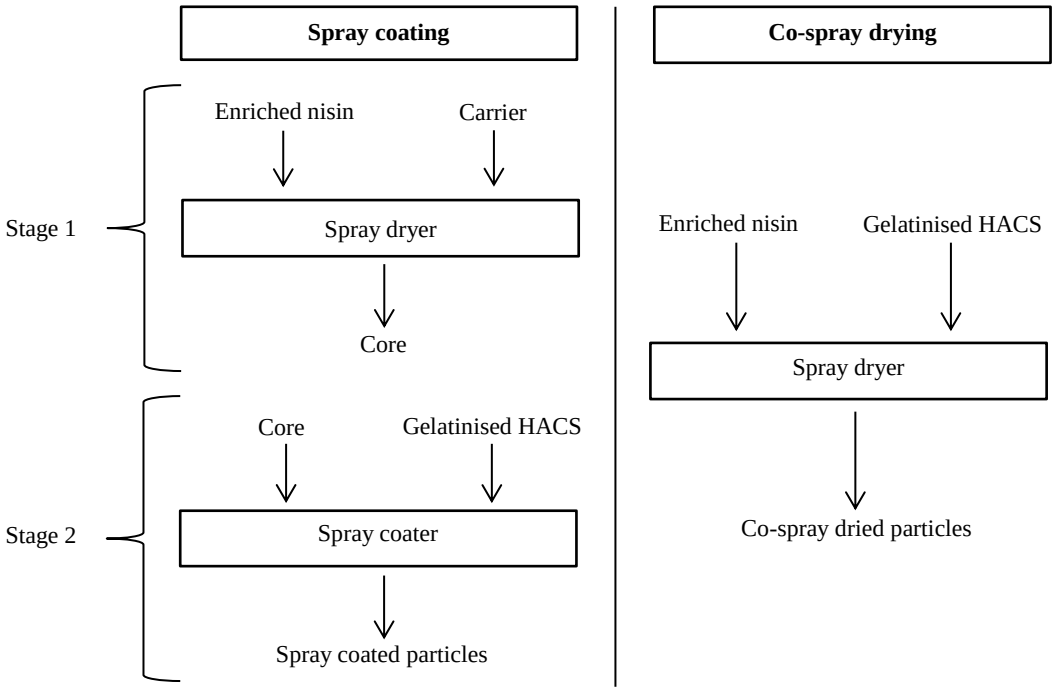

Fig. 4.1. Overview of the two entrapment procedures.

4.3. Materials and methods

4.3.1. Materials

All reagents and chemicals were procured from Sigma Aldrich (Arklow, Ireland) unless otherwise stated. The specific starch from Sigma Aldrich that was used was native high amylose corn starch (S4180). The specific enzymes from Sigma Aldrich that were used were porcine pancreatic α-amylase (A3176), bovine α-chymotrypsin (C4129), α-amylase from the bacterium *Bacillus subtilis* (10070) and β-amylase from barley (A7130).

4.3.2. Preparation of nisin

Nisin was enriched from a commercial nisin preparation (Nisaplin®, DuPont, Beaminster, UK) by salting out (Gough et al., 2017) as described in Chapter 2. This is referred to in the text as enriched nisin.

4.3.3. Preparation of gelatinised starch

Gelatinisation was attempted on high amylose corn starch (HACS) by heat, pH and shear treatments. All samples were prepared at 5% solids with the exception of the samples that underwent pressure cooker treatment; these samples were prepared to ensure the percentage solids were below 5% for the duration of the heat treatment. All heat treatments performed at 121 °C were for 40 min,

these were performed with the samples at pHs ranging from 1 to 12. Shear treatments were performed using a T25 Ultra-Turrax® (IKA, Staufen, Germany) at up to 15 min at maximum speed (25,000 rpm). Pressure cooker (Presto 1755, National Presto Industries, Eau Claire, US) treatments were performed at 120 °C for up to 74 cumulative h at that temperature, with periodic shutdowns to allow sampling and replacement of evaporated water. High heat treatment was performed in an Elbanton oil bath (Hettich Benelux B.V., Geldermalsen, Netherlands) at 140 °C for up to 2 h.

4.3.4. Production of cores for spray coating

Two spray dryers were used. A bench scale spray dryer (B-191, Buchi, Flawil, Switzerland) was used to produce cores with a range of compositions in order to ascertain optimal flowability. The optimised blend was then produced on a pilot scale spray dryer (Anhydro Laboratory Spray Dryer Size 3, SPX Flow Technology A/S, Soeborg, Denmark) in order to produce in a single batch a sufficient quantity of cores for all subsequent coating trials.

The standard conditions for producing the cores in the bench scale spray dryer were an air inlet temperature of 180 °C, and an outlet temperature of 92 °C and an airflow rate of 600 L/h. The range of inlet temperature, outlet temperature and total solids used were 120 to 180 °C, 80 to 99 °C and 5 to 40%, respectively. Cores were produced containing lactose and lactose blended with trehalose (Treha™, Cargill, Manchester, UK) or maltodextrin (dextrose equivalent (DE) 6 and DE 12) (Roquette, Corby, UK) at up to 50% w/w. Cores were also produced containing whey protein isolate (WPI) (Isolac®, Carbery, Cork, Ireland) blended with a

commercial nisin preparation and enriched nisin perpetration at up to 95% (w/w) WPI. Cores containing only WPI, skimmed milk powder and Nisaplin® were produced for comparison. The optimised cores comprised a WPI/enriched nisin blend of which 0.5% w/w was pure nisin; this was suspended at 15% w/w total solids and spray dried using a pilot scale spray dryer (Anhydro Laboratory Spray Dryer Size 3, SPX Flow Technology A/S, Soeborg, Denmark) with an air inlet and outlet temperature of 180 °C and 90 °C respectively.

4.3.5. Spray coating

To spray-coat the WPI/enriched-nisin cores with the coating solution a VFC-LAB Micro Flo-Coater (Freund-Vector, Iowa, US) was used. Spray coating was performed using the Wuster process. The optimised conditions were as follow: a 17 cm inner partition and the type 5 air distribution plate were used, the inlet air temperature was 95 °C, the nozzle airflow temperature was 90 °C and the coating solution was kept at 80 °C before being sprayed on the sample to prevent premature retrogradation, the coating feed rate was 1.1 mL/min, the main airflow was initially 40 LPM and the atomising air pressure was initially 400 mBar which were increased over the course of the coating run to 50 LPM and 500 mBar respectively in order to maintain good particle flow and 10 g of the WPI/enriched nisin cores was loaded into the machine. The cores were coated in 15 coating cycles. Each of these coating cycles consisted of 2 min of coating and 30 s without coating (to allow the turbulence break up agglomerates and ensure the coating layers were dried). Every 5 cycles the coating was pumped back to its heated source bottle to maintain its temperature at 80 °C.

4.3.6. Co-spray drying

For entrapment by co-spray drying, enriched nisin was blended with gelatinised starch such that the total solids were 5% and the pH of the solution was 4.75. The solution was kept at 80 °C before spray drying to prevent retrogradation. This was spray dried on a B-191 spray dyer (Buchi, Flawil, Switzerland) with an air inlet temperature of 180 °C, and an outlet temperature of 92 °C and an airflow rate of 600 L/h.

4.3.7. Simulated digestion

To determine the resistance of the HACS based delivery systems to digestion during gastrointestinal transit two simulated digestion approaches based on simplified versions of the INFOGEST method (Minekus et al., 2014) were used. Digestion of spray coated and co-spray dried particles for microscopy and particle sizing was based on the small intestine stage and involved a digestion with pancreatic α-amylase at a concentration of 200 U per mL in a solution comprising 55.5 mM Cl^-, 0.6 mM Ca^{2+} and 20 mM KH_2PO_4, at pH 7 which was incubated at 37 °C for 2 h and the digesta comprised 6% w/w of the total mass (0.6 g in a final volume of 10 mL). Digestion for insoluble solids, which was performed on the co-spray dried particles, comprised an additional initial incubation at pH 3 for 2 h (gastric stage) and at the small intestine stage the inclusion of α-chymotrypsin at 25 *N*-benzoyl-$_L$-tyrosine ethyl ester (BTEE) U per mL in addition to the pancreatic α-amylase.

4.3.8. Breakup of retrograded HACS by enzymatic and chemical approaches

To quantify the nisin entrapped within the HACS based delivery systems it was necessary to break up the retrograded HACS matrixes. Enzymatic digestions utilising α-amylase from the bacterium *B. subtilis* and β-amylase from barley (separately and sequentially) were performed at a ratio of 0.25 mg of enzyme per mg of substrate and at a pH appropriate to the enzymes at 37 °C for up to 72 h, additionally pancreatic α-amylase was utilised under the same conditions for comparison purposes. Dimethyl sulfoxide (DMSO) was used to solubilise retrograded HACS as per the method of (Han & Lim, 2004) and the solubilised nisin and HACS were separated as per the method of (Xiao et al., 2010)**.**

4.3.9. Reversed-phase high performance liquid chromatography

The concentration of nisin was determined using reversed-phase high performance liquid chromatography (RP-HPLC). RP-HPLC was carried out using a Waters e2695 separation module with a Waters 2489 UV/visible detector, running on Waters Empower software (Waters, Dublin, Ireland) and a Jupiter 5 µm C18 300A 250 mm × 4.6 mm from Phenomenex (Macclesfield, UK). Solvent A was 0.1% (v/v) trifluoroacetic acid (TFA) in Milli-Q water (Merck Millipore, Carrigtwohill, Ireland), and solvent B was 90% (v/v) HPLC-grade acetonitrile (Thermo Fisher Scientific, Dublin, Ireland) containing 0.1% TFA (v/v) in Milli-Q water. Flow rate was 1.0 mL/min. Each sample was run as follows: 22.2% solvent B for 5 min, a

gradient increase from 22.2% B to 55.6% B over 30 min, a 2 min gradient increase from 55.6% B to 100% B, 5 min at 100% B, 2 min gradient decrease to 22.2% B, 5 min at 22.2% B. Nisin was detected by absorbance at 214 nm. The nisin peak appeared between 24 and 25 min which corresponded to approximately 36% acetonitrile. Nisaplin® was used to generate a standard curve and the amount of nisin was calculated from the area of the peaks at 214 nm.

4.3.10. Physical characterisation

Moisture content was measured using dry weight differences according to published methods (GEA Niro, 2006b). Flowability was measured using the time taken for a defined volume of powder to leave a rotating drum, in accordance with a published method (GEA Niro, 2006a).

Visual structural and coating analysis was performed using an Olympus BX51 light microscope (Olympus BX-51, Olympus Corporation, Tokyo, Japan) under a 20× dry objective lens using both differential interference contrast (DIC) and bright field setup. Images were taken using a ProgRes® CT3 camera in conjunction with ProgRes® CapturePro version 2.10.0.0 software (Jenoptik, Jena, Germany). To identify the starch component of samples, iodine stain (1% iodine and 2% potassium iodine) was added such that it made up 10% of the total volume of the samples. To inhibit agglomeration of sample components, glycerol was added such that it made up 10% of the total volume of the samples.

Particle size was determined using a Mastersizer 3000™ and a Morphologi® G3 (Malvern Instruments, Malvern, Worcestershire, UK). For the Mastersizer 3000™ a Hydro SV (Malvern Instruments, Malvern, Worcestershire, UK) wet

sample dispersion unit was used and the optical settings were a particle refractive index of 1.45 and a dispersant refractive index of 1.33. For the Morphologi® G3 analysis, sample powder volumes of 11 mm^3 and the 2.5× objective lens were used.

4.3.11. Biological activity assay

Biological activity was estimated by agar diffusion activity assays (Ryan, Rea, Hill, & Ross, 1996). *Lactococcus lactis* subsp. *cremoris* HP, the indicator strain, was grown overnight in M17 broth (Oxoid, Basingstoke, UK) containing 0.5% lactose (VWR, Dublin, Ireland) (LM17). LM17 agar was tempered to 45 °C and seeded with 0.5% of the indicator strain. The seeded agar was dispensed in 20 mL aliquots into sterile petri dishes, these were allowed to solidify and wells of 5 mm in diameter were bored in the agar. Serial two-fold dilutions of the samples were performed in a 50 mM lactic acid buffer, pH 3.5. The samples (50 µL) were dispensed into the wells and the plates incubated overnight at 30 °C. The activity of nisin resulted in zones of inhibition surrounding the wells. The minimum inhibitory concentration (MIC) (µg/mL) was calculated by plotting the area of the zone of inhibition at each dilution stage against the log of the nisin concentration (Bernbom et al., 2006); these had a linear relationship and the MIC was calculated from the equation of the line. The MICs were tested for significant difference by the Kruskal-Wallis test using the SigmaStat software (Systat Software, San Jose, US).

4.3.12. Insoluble solids

Intact retrograded starch is insoluble in water (Kapelko-Żeberska, Zięba, & Singh, 2015), however the oligosaccharides produced by α-amylase digestion are water soluble (Sundarram & Murthy, 2014) and thus the relative resistance to digestion of each of the blends produced by co-spray drying could be inferred from their insoluble solids.

Total insoluble solids were measured in co-spray dried samples that had undergone simulated digestion and undigested controls. Each sample was centrifuged at 179×*g* for 5 min and the supernatant was removed. Then the sample was suspended in 10 mL H_2O and centrifuged at 403×*g* for 5 min and the supernatant was removed. The last step was repeated a further two times to ensure the entire soluble component was removed. The insoluble component was transferred to preweighed discs and dried in a Gallenkamp OVA031 oven (Weiss Technik, Loughborough, UK) overnight.

4.3.13. Entrapment efficiency

The efficiency of the entrapment was tested based on published methods (Hong, Lee, Baek, & Choi, 2012; Saboktakin, Tabatabaie, Maharramov, & Ramazanov, 2011). Twenty mg of co-spray dried particles were suspended in water to a final volume of 10 mL, mixed on a roller for 10 min, centrifuged at 1,000×*g* for 5 min and the supernatant containing the non-entrapped nisin was collected. The pellet was resuspended in 10 mL and the previous steps were repeated. The nisin was quantified by RP-HPLC.

4.4. Results and discussion

4.4.1. Preparation of gelatinised starch

Gelatinisation temperature and time used for HACS when it is used as a coating materials have ranged from 120 to 160 °C and from 15 to 120 min (Desai, 2007; Dimantov et al., 2004; Krogars et al., 2003; Recife, Meneguin, Cury, & Evangelista, 2017) and the temperature for complete gelatinisation of HACS has been reported as 129 °C (Ai & Jane, 2015). It is known that the degree and temperature required for gelatinisation can be effected by shear treatments on the starch granules and by the pH and the length of time gelatinisation takes place over (Alcázar-Alay & Meireles, 2015; Baks, Bruins, Janssen, & Boom, 2008; Wang, Truong, & Wang, 2003). Starch was gelatinised by a range of pH, shear and heat treatments, a selection of which are shown in Fig. 4.2. The degree of gelatinisation was examined by light microscopy. Shear, pH, and long term heat treatments resulted in a minor increase in the degree of gelatinisation (Fig. 4.2). It was noted that the fastest retrogadation occurred in samples at pH 4.5 and 5. From a production point of view it would be preferable to use a lower temperature treatment, however only the high heat treatment (140 °C) for 2 h gave complete gelatinisation which would be necessary to have a homogenous solution appropriate for coating with.

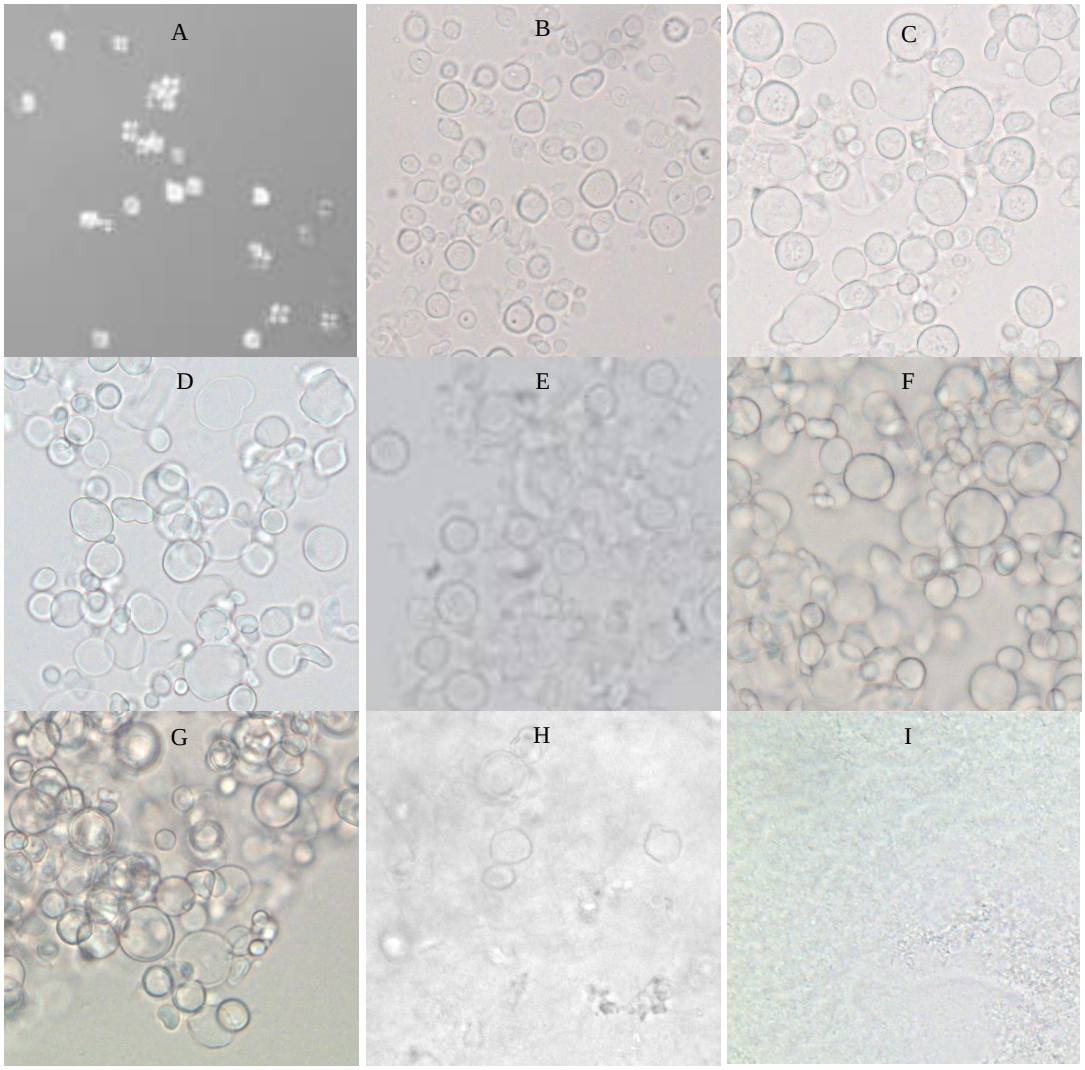

Fig. 4.2. Selected attempts at gelatinisation of high amylose corn starch (HACS). Untreated HACS (A), HACS heat treated (121 °C for 40 min) at pH 1.5, 5 and 12 and in conjunction with shear treatment (B, C, D and E respectively), HACS heat treated (120 °C) for 5 h (F), HACS heat treated (120 °C) for 74 h (G), HACS heat treated (140 °C) for 1 h and 2 h (H and I respectively). All images were taken at 20X magnification using a differential interference contrast (DIC) setup apart from image A, which was taken using bright field to obtain the characteristic extinction cross of untreated starch granules.

4.4.2. Production of cores

A range of carriers were used to produce cores of suitable flowability (Fig. 4.3). The yield when producing the cores, is a reflection of the flowability of the cores (Yang, Xu, Qu, & Li, 2015), with the higher the yield, the better the flowability. There were distinct differences in yield between carrier type while carriers of the same constituents tended to have similar yield irrespective of the ratio

in the blend or the process conditions. The most flowable carrier was the WPI and whereas the salt in the commercial nisin preparation likely reduced its flowability when blended with the WPI, the lower salt in the enriched nisin preparation allowed good flow when combined with the WPI. That no greater yield than ~60% was achieved on the bench scale spray dryer is likely due to the limitations of bench scale spray drying (Maury et al., 2005; Soares e Silva et al., 2012). As expected the moisture content of the products were directly related to the outlet temperature of the spray dryer and by keeping the outlet temperature above 90 °C, a moisture content below 5% could be achieved.

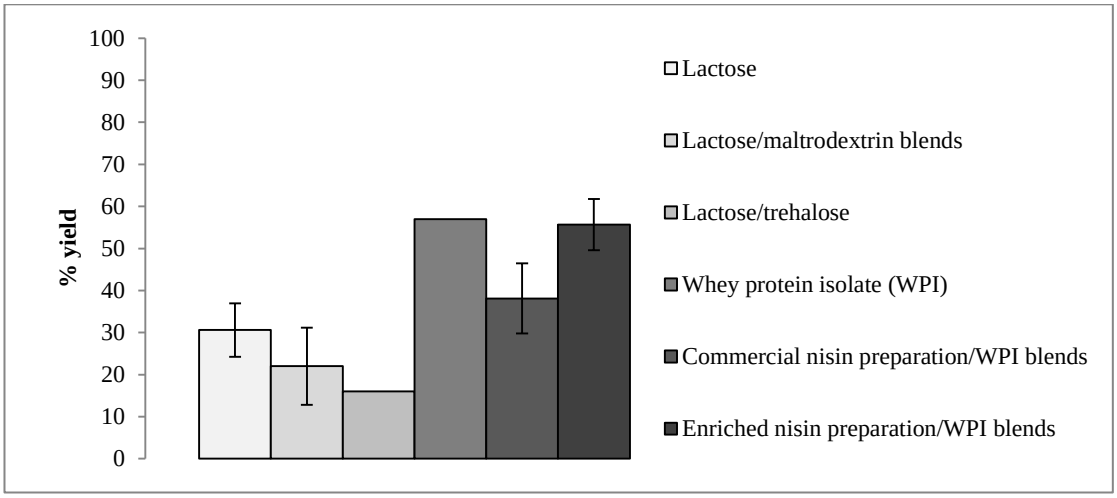

Fig. 4.3. Yields of potential carrier materials and cores for spray coating when produced on bench scale spray dryer. The carrier yields are average by constituents irrespective of constituent blend or process conditions. Mean values are ± standard deviation, $n \geq 3$.

The feed stock of WPI/enriched nisin cores for spray coating was produced in a single batch using a pilot scale spray dryer. The cores contained 0.5% nisin and had a mean diameter of 27.6 µm (volume moment mean, D(4,3)). There was no significant difference between the antibacterial activity of nisin in the commercial product and in the spray dried cores; the activity of nisin was not affected by spray drying. Quantification by RP-HPLC was not possible due to the nisin and

components of the WPI co-eluting from the RP-HPLC column. Regarding flowability; when tested by GEA Niro analytical method A 23 (GEA Niro, 2006a), the WPI before spray drying had a flowability (± SE) of 19.3 s (±0.4, n=3) which compares favourably to the WPI/enriched nisin cores after spray drying (19.7 ±0.3 s, n=3), indicating that spray drying did not negatively impact on flowability. Although these values are poorer than the flowability of SMP (14 ±0.4 s, n=4), the flow behaviour of the WPI and the WPI/enriched nisin cores was considerably better in the spray coater. This superior aerosolisation is likely due to the air pockets that are visible on the surface of the cores (Fig. 4.4) as pores improves the aerosolisation of a powder (Vanbever et al., 1999).

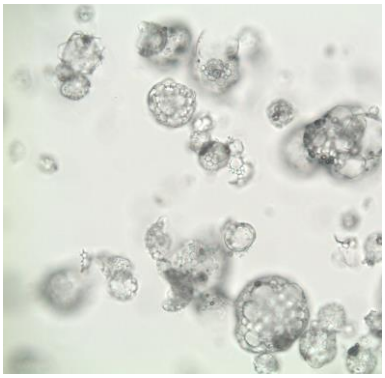

Fig. 4.4. WPI/enriched nisin cores for spray coating viewed by bright field at 20X magnification.

4.4.3. Spray coating

The spray coating procedure was optimised to produce a product with the greatest possible coating thickness. Although the irregular surface of the core was critical to the cores obtaining good flowability, this created a challenge to achieve complete coating of the core as irregular surfaces are known to inhibit the spread of coating solution over the core (Asija, 2012). There were three key areas where a coating run could fail: firstly particles could become trapped in the top of the spray

coater, secondly the coating solution could dry too slowly on the particle surface resulting in agglomerates and thirdly the coating solution could retrograde into a solid gel in the tubing and nozzle.

By adjusting the process parameters to reduce the likelihood of one source of failure, could increase the likelihood of another. For example, increasing the pump speed to reduce the likelihood of the coating solution gelling in the tubing/nozzle also increased the likelihood of agglomerates in the coating chamber whereas increasing the solids content of the coating solution allowed for faster drying and thus reduced formation of agglomerates but also increased the likelihood of the coating solution gelling in the tubing/nozzle. Another example is the main and coating nozzle airflow, an increase in which reduced the likelihood of agglomerates forming while increasing the likelihood of particles becoming trapped in the top of the coating chamber. A detailed overview of the range of process conditions trialled and the optimum balance between them is described in Table 4.1. The coated product is seen with the HACS coating visible by iodine staining in Fig. 4.5A and B. This demonstrates spray coating of <100 μm diameter cores with HACS that retrogrades *in situ* is possible.

Table 4.1. Optimisation of spray coating procedure.

Variable	Effect	Range tested	Optimum
Preheating of equipment	Reduces stickiness to chamber wall	Temperature levels off after 30 min	30 min
Mass of cores for coating	Too great resulted in lag in bottom of chamber during recirculation	5 to 50 g	10 g
Air distribution plate	Recirculation of the particles	Full range of plates	Type 5 air distribution plate
Main Airflow	↑ Particles will lodge in the top of chamber ↓ Risk of particle agglomeration (coating will dry too slowly)	27 to 100 L/min	40 L/min increasing towards 50 L/min over the course of the run
Coating nozzle airflow	↑ Particles will lodge in the top of chamber ↓ Risk of particle agglomeration (droplet size will be too large)	125 to 1500 mBar	400 mBar
Coating solution temperature	Reduce coating solution viscosity	45 to 90 °C	80 °C
Coating solution concentration	Viscosity and rate of retrogradation increase with concentration	1 to 10% (w/w)	5% (w/w)
Inlet temperature	Drying rate of coated particles	50 to 100 °C	95 °C
Nozzle airflow temperature	Drying rate of coated particles	0 to 90 °C	90 °C
Pump speed	↑ Too much solution enters the chamber causing agglomeration ↓ Coating solution resides too long in tubing and nozzle and coating solution retrograding within them	10 to 100 rpm	20 rpm
Wurster partition	Recirculation of the particles	6.4 to 17 cm	17 cm
Filter pulse interval	Dislodging particulates from upper chamber	1 to 5 s	1 s
Spray cycle	Longer spray time: ↑ Risk of particle agglomeration ↓ Risk of coating solution retrograding within nozzle and tubing	1 to 8 min	2 min
	Longer interval time: ↓ Risk of particle agglomeration ↑ Risk of coating solution retrograding within nozzle and tubing	10 s to 2 min	30 s
	Number of spray cycles before coating solution is returned to stock container and number a times this is repeated (cycle groups): ↓ Risk of particle agglomeration ↓ Risk of coating solution retrograding within nozzle and tubing, however each time this procedure is performed the risk of nozzle clogging increases	1 to 21 cycles and 3 to 5 cycle groups	3 groups of 5 cycles each

To test their susceptibility to small-intestinal digestion, the products of spray coating underwent a 2 h digestion with pancreatic α-amylase. This appears to have removed the HACS coating as they were no longer stainable with iodine (Fig. 4.5C and D) and the size of the particles reverted to a similar size distribution to that of the WPI carrier (Fig. 4.6). The enzymatic resistance of HACS coatings are related to their thickness (Dimantov et al., 2004). It was not possible to increase the coating thickness without the coating run failing as described previously. Additionally, as this approach required that each layer of the coating be solidified before the next layer was applied, there was negligible possibility of crosslinking between layers. To circumvent these problems a co-spray drying approach was employed so that all the HACS in the particle could retrograde simultaneously and thus achieve the thickness required to resist small-intestinal digestion.

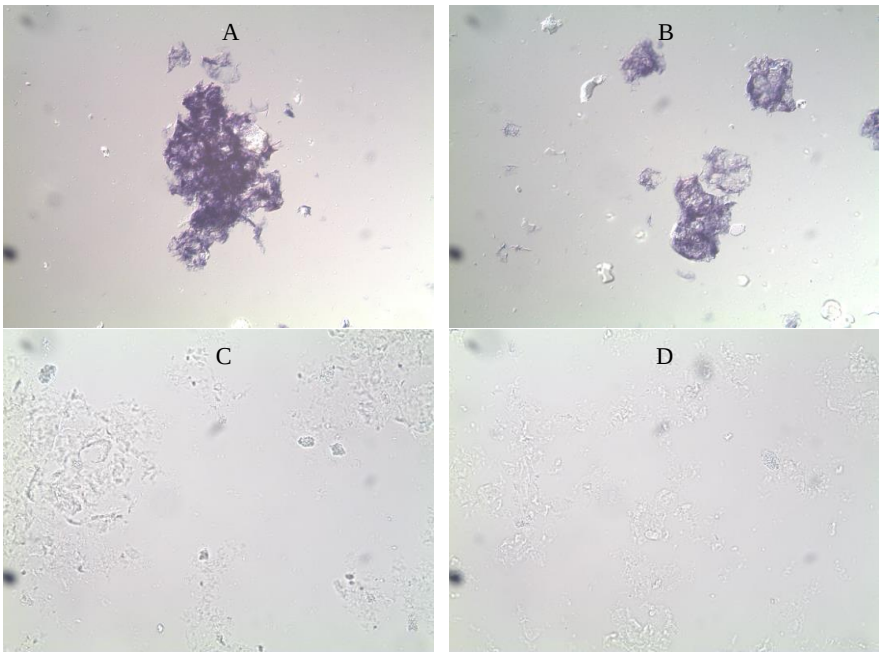

Fig. 4.5. Images of spray coated samples before (A and B) and after (C and D) digestion with pancreatic α-amylase. Samples iodine stained and viewed by differential interference contrast (DIC) at 20X magnification.

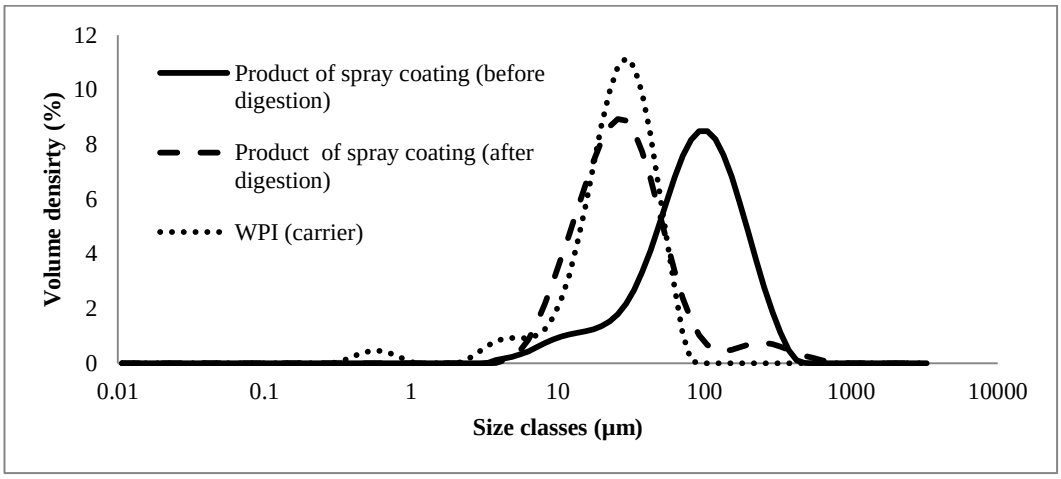

Fig. 4.6. The effect of digestion with pancreatic α-amylase on the products of spray coating by way of change in particle size. The WPI used in production of the cores is shown as a comparison.

4.4.4. Co-spray drying

As the most rapid retrogradation of the HACS had previously occurred at pH 4.75, spray drying was performed this pH. A total solids of 5% w/w was the greatest concentration of HACS that had a viscosity suitable for bench scale spray dryer. The resulting powder had a circle equivalent (CE) mean diameter of 10.9 µm, which is consistent with the known particle sizes produced by the make and model of spray dryer used (Maury et al., 2005).

A spray dried HACS powder was digested with pancreatic α-amylase in the same manner as the spray coated sample. As spray dried HACS successfully demonstrated a degree of resistance to digestion (Fig. 4.7), it was attempted to entrap the enriched nisin in HACS by co-spray drying. These were then subjected to a more thorough simulated digestion comprising a gastric stage of 2 h at pH 3 followed by an intestinal stage of 2 h at pH 7 in the presences of α-chymotrypsin and pancreatic α-amylase.

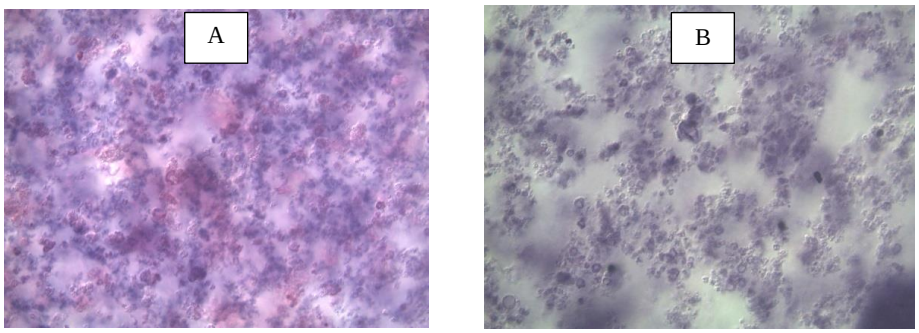

Fig. 4.7. Spray dried HACS before (A) and after (B) digestion with pancreatic α-amylase. Samples iodine stained and viewed by differential interference contrast (DIC) at 20X magnification.

As the change is particle size due to digestion of the co-spray dried HACS/nisin blends could not be accurately measured on the Mastersizer 3000™ due to the formation of agglomerates, the relative resistance of the different blends were inferred from their total insoluble solid, as intact retrograded starch is insoluble in water (Kapelko-Żeberska et al., 2015) whereas the oligosaccharides produced by α-amylase digestion are water soluble (Sundarram & Murthy, 2014). As expected the greater proportion of HACS in the blend the greater the resistant to simulated digestion of spray dried nisin/starch (Fig. 4.8), however it is noticed that the 100% enriched nisin sample increased in insoluble solids after digestion, implying that some of the digestion products of nisin have lower solubility than intact nisin (Fig. 4.8).

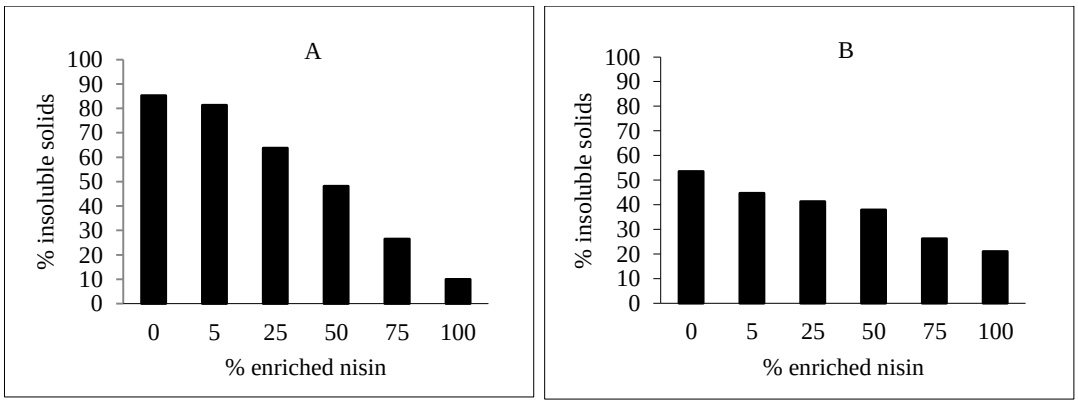

Fig. 4.8. Percentage insoluble solids in co-spray dried HACS/enriched nisin blends before (A) and after (B) simulated digestion.

In order to quantify the intact nisin within the products of co-spray drying after simulated digestion, a way of breaking apart the products of co-spray drying that would not denature the nisin was investigated. However amylases from bacterial and plant sources had no greater ability to digest retrograded HACS than the porcine pancreatic amylase used in the INFOGEST method and although it was possible to solubilise retrograded HACS in DMSO and then precipitate it out of solution using ethanol, nisin was not detected in the sample in subsequent RP-HPLC analysis.

When the entrapment efficiency of the samples were tested, no nisin was found to be bound in the samples. Aqueous conditions induce a degree of swelling and porosity in starch gels (Chourasia & Jain, 2003; McConnell et al., 2007), this was likely how nisin was released and this problem was likely exacerbated by the very small size of the particles produced by co-spay drying. Using a much larger particle could circumvent this problem.

4.5. Conclusions

In this study it was attempted to achieve colonic delivery of nisin in particles with a diameter <100 μm through the use of only starch (HACS) and water, by protecting the nisin from digestion during gastrointestinal transit. By using a WPI carrier it was possible to achieve flowability suitable for spray coating with cores <100 μm in diameter, however while the porosity of the carrier increased the flowability of the cores it also reduced the coating efficiency. To fully gelatinise HACS such that it was a homogenous solution suitable for coating required a heat treatment of 140 °C for 2 h. This coating solution was successfully applied to the

cores such that the coating retrograded into a solid gel on the particle surface. The spray coated HACS coating did not resist digestion, this is likely because it was too thin and having each coating layer retrograde separately reduced the crosslinking potential of the coating. Co-spray drying resulted in particles in which all the HACS could retrograde simultaneously and these particles possessed a capacity to resist digestion, however they did not retain nisin. The ability of these particles to resist digestion shows the potential of this approach, using a larger particle could address the issue with nisin retention.

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Chapter 5

Entrapment of nisin in a starch gel and fermentation of starch by *Ruminococcus bromii*

5.1. Abstract

Oral delivery is the most desirable route of delivery for bioactive proteins and peptides; however digestion during gastrointestinal transit can remove the potential health benefits of these bioactives. Entrapment is often employed for the oral delivery of bioactives, particularly for delivery to the colon. The majority of current approaches come from a pharmaceutical perspective and thus, have aspects that would be less than ideal for a food product such as complex entrapment procedures and ingredients that are not clean-label.

Starch that resists digestion in the upper gastrointestinal tract but can be digested by bacteria in the colon, known as 'resistant starch', is a potential entrapment material; high amylose corn starch (HACS) is particularly high in resistant starch. Heating and cooling starch in water causes the dissociation and reassociating of the component amylose and amylopectin chains of starch, in a process known as gelatinisation and retrogradation, which can result in a solid starch gel. In this study the antimicrobial peptide nisin was used as a model bioactive peptide.

By gelatinising and retrograding HACS in the presence of nisin it was possible to entrap nisin in starch. Nisin losses during processing (115 °C for 15 min) were 1.59% (± 0.04, n = 3) and of the original nisin 49.64% (± 1.79, n = 3) was not bound to the gel; this implies that ~49% of the nisin was entrapped. To quantify the entrapped nisin, fermentation of the HACS with the starch digesting bacteria *Ruminococcus bromii* was used as a means of releasing the nisin from the starch. Complete fermentation was not achieved although a greater % of the HACS was fermented than in previously published work. For simulated *in vitro* digestion of the

HACS/nisin gel a chewing model (mean particle diameter: 1.8 ± 0.2 mm) was coupled with a modified version of the *in vitro* INFOGEST digestion method. After simulated digestion there was less nisin detected in solution in the samples with a HACS/nisin gel compared to the controls. This implies that a portion of the entrapped nisin remained bound in the HACS gels and that the HACS gel would allow nisin to reach the colon. However this could not be conclusively stated, as nisin is unstable at the pH of the small intestine (pH7), which impairs accurate measurement of how much is released from the HACS gel.

5.2. Introduction

Bioactive proteins and peptides have numerous health benefits, however their bioavailability can be limited if taken orally as they can be digested during gastrointestinal transit, particularly due to the low pH and pepsin in the stomach, and the trypsin, chymotrypsin and carboxypeptidase in the small intestine (Goodman, 2010; Segura-Campos, Chel-Guerrero, Betancur-Ancona, & Hernandez-Escalante, 2011).

Delivering peptides to the colon is of particular interest due its lower levels of proteases and higher responsiveness to several permeation enhancers (Chen et al., 2017; Fetih et al., 2006; Maroni et al., 2012; Petersen et al., 2013). Additionally colonic delivery is essential for bioactive peptides that have a local effect in the colon such as antimicrobial peptides.

Antimicrobial peptides, particularly the bacterially produced class known as bacteriocins are gaining interest as an alternative to antibiotics due to the rise in antibiotic resistance (Allen, Trachsel, Looft, & Casey, 2014). Many bacteriocins

have a narrow spectrum of activity that allows treatment of gastrointestinal infections without disrupting the native bacteria (Cotter, Ross, & Hill, 2013). Many bacteriocins are heat stable (Collado et al., 2005; Deraz et al., 2005; Oh, Kim, & Worobo, 2000) which allows the possibility of high heat processing conditions.

Nisin is a bioactive peptide that has activity against a range of Gram positive bacteria and is widely used as a food preservative (Abee & Delves-Broughton, 2003). Nisin is highly heat stable and when at pH 3 it can be heated to 115 °C for 20 min with <5% loss in activity (Davies et al., 1998). If taken orally, nisin is digested during gastrointestinal transit (Younes et al., 2017), which makes it an ideal candidate for testing a colonic delivery system. Nisin has been proposed as a model bioactive peptide for colonic delivery systems due to its commercial availability and thorough characterisation (Habib & Sakr, 1999; Mallen, 2017; Ugurlu, Turkoglu, Gurer, & Akarsu, 2007).

Starch that is resistant to digestion in the upper gastrointestinal tract, but can be digested by bacteria in the colon is termed ‘resistant starch’ (Sajilata, Singhal, & Kulkarni, 2006). Resistant starch has been proposed as a protective coating that could provide colonic delivery (Basit, 2005). The portion of starch that resists digestion is determined by starch source and type; in this study high amylose corn starch (HACS) (70% amylose starch from maize) was used, which contains 46% resistant starch on an w/w basis (McCleary, McNally, & Rossiter, 2002).

Starch is comprised of the carbohydrate polymers amylose and amylopectin. In plants amylose and amylopectin are arranged in a semi-crystalline form known as a starch granule. When these granules are heated in the presence of water the amylose and amylopectin disassociate, with the granules leaching amylose and absorbing water causing them to swell and ultimately dissipate. When the solution is

subsequently cooled, the amylose and amylopectin re-associate, turning the solution into a starch gel, with the gel strength primarily determined by amylose content. These two stages are referred to as gelatinisation and retrogradation (Alcázar-Alay & Meireles, 2015; Wang et al., 2015).

In Chapter 4 nisin was entrapped in HACS using spray coating and co-spray drying, to protect it from digestion during gastrointestinal transit and enable it to be delivered to the colon. The products of spray coating approach had negligible digestion resistance. The products of the co-spray drying approach possessed digestion resistance however they had negligible entrapment efficiency. It was suggested in Chapter 4 that the poor entrapment efficiency of the products of co-spray drying was related to their small particle size (diameter ≈ 10 μm). In this Chapter, to entrap nisin in larger particles, nisin's high heat stability was utilised. To entrap the nisin in a HACS gel for colonic delivery, HACS was blended with nisin and then the HACS was gelatinised and retrograded forming a solid gel.

The approach in this study differs from previous studies in two ways. Firstly the entrapment material compromises of only starch and water, which makes it suitable for a 'clean-label' approach; this is in contrast to many of the previous studies that have used a starch based coating that incorporate ingredients such as ethyl cellulose, triacetin, polymethacrylate and triethyl citrate (Freire et al., 2010; McConnell et al., 2007; Milojevic et al., 1996; Pu et al., 2011; Wilson & Basit, 2005).

A second way in which the approach in this study is novel is the simplicity of the entrapment procedure. Most starch based entrapment procedures use compression coating, spray coating or co-spray drying to apply a starch coating (Desai, 2007; Desai, 2005; Dimantov, Greenberg, Kesselman, & Shimoni, 2004;

Freire et al., 2010; McConnell et al., 2007; Milojevic et al., 1996; Moussa & Cartilier, 1997; Palviainen et al., 2001; Pu et al., 2011; Recife, Meneguin, Cury, & Evangelista, 2017; Wilson & Basit, 2005). These are in contrast to the approach used in this study whose simplicity may make it easier to use in commercial applications.

5.3. Materials and methods

5.3.1 Materials

All reagents and chemicals were procured from Sigma Aldrich (Dublin, Ireland) unless otherwise stated. The specific starch from Sigma Aldrich that was used was native high amylose corn starch (S4180). The specific enzymes from Sigma Aldrich that were used were porcine pancreatic α-amylase (A3176), bovine α-chymotrypsin (C4129) and salivary amylase (A1031).

5.3.2. Preparation of nisin

Nisin was enriched from a commercial nisin preparation (Nisaplin®, DuPont, Beaminster, UK) by salting out (Gough et al., 2017) as described in Chapter 2. This is referred to in the text as enriched nisin.

5.3.3. Reversed-phase high performance liquid chromatography

The concentration of nisin was determined using reversed-phase high performance liquid chromatography (RP-HPLC). RP-HPLC was carried out using a Waters e2695 separation module with a Waters 2489 UV/visible detector, running on Waters Empower software (Waters, Dublin, Ireland) and a Jupiter 5 µm C18 300A 250 mm × 4.6 mm from Phenomenex (Macclesfield, UK). Solvent A was 0.1% (v/v) trifluoroacetic acid (TFA) (Sigma-Aldrich, Arklow, Ireland) in Milli-Q water (Merck Millipore, Carrigtwohill, Ireland), and solvent B was 90% (v/v) HPLC-grade acetonitrile (Thermo Fisher Scientific, Dublin, Ireland) containing 0.1% TFA (v/v) in Milli-Q water. Flow rate was 1.0 mL/min. Each sample was run as follows: 22.2% solvent B for 5 min, a gradient increase from 22.2% B to 55.6% B over 30 min, a 2 min gradient increase from 55.6% B to 100% B, 5 min at 100% B, 2 min gradient decrease to 22.2% B, 5 min at 22.2% B. Nisin was detected by absorbance at 214 nm. The nisin peak appeared between 24 and 25 min which corresponded to approximately 36% acetonitrile. Nisaplin® was used to generate a standard curve and the amount of nisin was calculated from the area of the peaks at 214 nm.

5.3.4. Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI TOF MS)

MALDI TOF MS was performed using an Axima TOF^2 mass spectrometer (Shimadzu Biotech, Kyoto, Japan) as previously described (Field et al., 2012).

5.3.5. Production of starch gels

Starch gels were prepared by heating high amylose corn starch (HACS). Blends ranged from 50 to 70% dilute HCl (w/w), with the balance made up by HACS and enriched nisin with the enriched nisin powder comprising less than 10% (w/w) of the total solids and the blends had a final pH of 3. These were heated at temperatures ranging from 121 to 115 °C for 15 min and subsequently incubated at 4 °C for a minimum of 16 h to ensure thorough retrogradation.

5.3.6. Simulated chewing

Two approaches were used to mimic the breakup of the gels due to chewing. The first approach was performed using an Eddingtons Mincer Pro (86002, Eddingtons, Hungerford, UK) as per the INFOGEST method (Minekus et al., 2014) using the 'fine' extrusion disk (apertures are ~4 mm in diameter) and in cases where a mincer was unsuitable such as when material had to be prepared under sterile conditions for use in fermentation vessels, the gel was broken using a sterilised spatula to an equivalent size to that of the mincer.

To more accurately model the effect of chewing a second approach was applied based on a 'chew and spit' approach (Wickham, Faulks, & Mills, 2009). The size of particles produced by chewing a HACS gel was determined by taking images of chewed particles using an Epson V700 scanner (Seiko Epson, Hemel Hempstead, UK) and measuring the equivalent spherical diameter of the particles using the Image J software (version 1.48) (Schneider, Rasband, & Eliceiri, 2012). Subsequently when preparing samples for digestion, they were chopped until they

reached the established diameter of 1.8 (± 0.2) mm, which was confirmed using the Epson V700 scanner and Image J software.

5.3.7. Digestion

There were two simulated digestion approaches based on the INFOGEST method, a recently developed standardized static method for the digestion of food (Minekus et al., 2014).

The first digestion approach was used to determine the resistance of the HACS gels to digestion by determining total insoluble solids after digestion (this is the same as the final digestion procedure performed in Chapter 4): namely an incubation of 0.6 g of digesta in a 5 mL electrolyte solution comprising 55.5 mM Cl^-, 0.6 mM Ca^{2+} and 20 mM KH_2PO_4, at pH 3 for 2 h at 37 °C and then an incubation with pancreatic α-amylase at a concentration of 200 U per mL and α-chymotrypsin at 25 *N*-benzoyl-$_L$-tyrosine ethyl ester (BTEE) U per mL at pH 7 for 2 h at 37 °C and a final volume of 10 mL.

The second digestion approach was used to determine the amount of nisin released through digestion. This was performed as per the INFOGEST method (Minekus et al., 2014) without bile or enzymes other than α-amylase. This involved an oral, gastric and intestinal stage. The HACS gels contained 1, 2 or 4% enriched nisin. An example digestion is as follows: For the oral stage 5 g of HACS gel containing 0.1 g (2% w/w) enriched nisin or a control containing 0.1 g of enriched nisin that had undergone the same processing as the test solution (suspended in a pH 3 dilute HCl solution, heated at 115 °C for 15 min, incubated at 4 °C for 16 h), were suspended in simulated salivary fluid (SSF) and salivary amylase (75 U/mL in final

oral solution) to a total volume of 10 mL; this was incubated at 37 °C for 2 min. For the gastric stage, the sample pH was adjusted to 3 using dilute HCl to a total volume of 20 mL; this was incubated at 37 °C for 2 h. For the small intestinal stage the pH was adjusted to 7 using dilute NaOH and combined with simulated intestinal fluid (SIF) and pancreatic α-amylase at a concentration of 200 U per mL to a total volume of 20 mL. Control digestion for the effect of pH were also performed for stages of the same duration, temperature and mineral composition but all at a pH of 3 and without α-amylase as it would be inactivated at this pH. Samples were taken at the end of oral, gastric and small intestine phase and quantified by RP-HPLC.

5.3.8. Insoluble solids

Intact retrograded starch is insoluble in water (Kapelko-Żeberska, Zięba, & Singh, 2015), however the oligosaccharides produced by α-amylase digestion are water soluble (Sundarram & Murthy, 2014) and thus the relative resistance to digestion of each HACS gel could be inferred from their insoluble solids.

Total insoluble solids were measured in samples that had undergone digestion and undigested controls. Water was added to the samples to bring their volumes to 50 mL and they were centrifuged at 1,000×*g* for 5 min. The insoluble component was transferred to preweighed discs and dried in a Gallenkamp OVA031 oven (Weiss Technik, Loughborough, UK) overnight.

5.3.9. Entrapment efficiency

The efficiency of the entrapment was tested based on published methods (Hong, Lee, Baek, & Choi, 2012; Saboktakin, Tabatabaie, Maharramov, & Ramazanov, 2011) by suspending the particles in water with mild agitation on a rotor, pelleting them by centrifugation and analysing the supernatant by RP-HPLC. The standard conditions were suspension at 10% (w/w) for 5 min and centrifugation for 5 min at 200×*g*. This suspension and pelleting treatment was repeated 5 times with each supernatant quantified by RP-HPLC.

5.3.10. Microscopy

Samples were examined using an Olympus BX51 light microscope (Olympus BX-51, Olympus Corporation, Tokyo, Japan) under a 20× dry objective lens using bright field setup. Images were taken using a ProgRes® CT3 camera in conjunction with ProgRes® CapturePro version 2.10.0.0 software (Jenoptik, Jena, Germany).

5.3.11. Bacterial fermentation

Bacterial fermentation was performed with *R. bromii* (ATCC 27255, LGC Standards, London, UK) which was grown in M2GSC media (Miyazaki, Martin, Marinsek-Logar, & Flint, 1997; Ze, Duncan, Louis, & Flint, 2012) which contained per 100 mL: 45 mg K_2HPO_4, 45 mg KH_2PO_4, 90 mg $(NH_4)_2SO_4$, 90 mg NaCl, 9 mg $MgSO_4 \cdot 7H_2O$, 9 mg $CaCl_2$, 1 g bacto casitone (BD, Wokingham, UK), 0.25 g yeast extract (Merck, Carrigtwohill, Ireland), 0.4 g $NaHCO_3$, 0.2 g cellobiose, 0.2 g

glucose (VWR, Dublin Ireland), 0.2 g soluble starch, 30 mL clarified rumen fluid, 0.1 mg resazurin and 0.1 g cysteine hydrochloride. The media was sparged with CO_2 to remove the O_2. The *R. bromii* were cultured under strict anaerobic conditions at 37 °C. All HACS gels were prepared for fermentations by heating at 115 °C for 15 minutes and retrograded and broken up as described previously. Fermentations were performed in 7.5 mL of media with 0.125 to 0.8 g of HACS gel for up to 96 h on a tube rotator (444-0502, VWR, Dublin, Ireland). Media was either seeded with 100 µL of turbid culture the previous evening (stationary phase culture) or on the morning of the fermentation (freshly seeded culture). Fermentations were also performed with cell free supernatant from a stationary phase culture prepared by centrifugation at 5444×*g* for 20 min. Fermentations were also performed in Multifor bioreactor vessels (Infors, Bottmingen, Switzerland) with 5 g of HACS gel for 20 h at a pH of 6.89, the rotor at 100 rpm and with continual sparging with nitrogen. In these vessels the fermentation was performed with total volume of 200 mL of which 0.5, 5 or 100% was stationary phase culture and the balance was M2GSC media. In all cases the fermentations were performed at 37 °C under strict anaerobic conditions and control fermentations without the HACS gel or with uninoculated media were performed. The products of the fermentation were pelleted by centrifugation at 200×*g* for 10 min in the case of the 7.5 mL fermentation or 500×*g* for 2 h in the case of the 200 mL fermentation vessels, freeze dried (Virtis Advantage, SP Scientific, Gardiner, NY, US) and the change in mass was compared to the controls to calculate the percentage of HACS gel fermented.

5.4. Results and discussion

5.4.1. Initial gel entrapment and comparison with previous work

HACS gels prepared by heating a HACS/nisin blend at 121 °C for 15 min, retrograding at 4 °C and broken up using a mincer, were tested for their nisin entrapment and for the ability to resist digestion. The relative resistance to digestion of each HACS gel could be inferred from their insoluble solids as intact retrograded starch is insoluble in water (Kapelko-Żeberska et al., 2015), however the oligosaccharides produced by α-amylase digestion are water soluble (Sundarram & Murthy, 2014).

The ability of the gels to retain nisin (entrapment efficiency) was increased by increasing the % solids (w/w) in the formulation of the gels (Fig. 5.1A) and also by reducing % enriched nisin (w/w) in the formulation of the gels (Fig. 5.1B). The resistance to digestion, which was inferred from the % insoluble solids remaining after digestion, was increased by lowering the % enriched nisin (w/w) and increasing the % HACS (w/w) in the formulation of the gels (Fig. 5.1C). It is noted, as in Chapter 4, that the digestion products of the enriched nisin contribute to the insoluble solids (Fig. 5.1D), so the true difference in digestion resistance in Fig 5.1C is even greater than that inferred from the insoluble solids.

In Chapter 4 the co-spray dried particles had a degree of resistance to digestion, however they had negligible entrapment efficiency which was believed to be due to their small particle size (diameter ≈ 10 μm). The Eddingtons Mincer Pro produces particles of ~4 mm in diameter. As expected, these larger particles had greater entrapment efficiency.

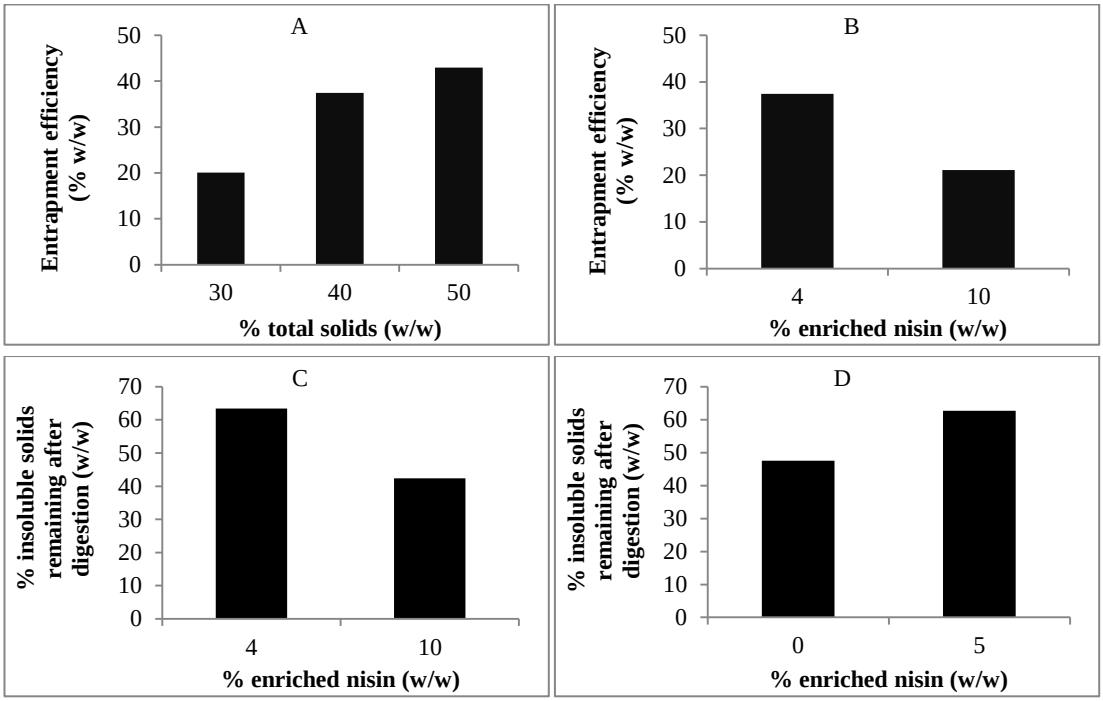

Fig. 5.1. Entrapment efficiency and digestion resistance of HACS gels. In section A all the gels were prepared with enriched nisin constituting the same % of total solids (10% w/w) and in sections B, C and D the gels were prepared with the same % total solids (40, 40 and 50% w/w, respectively).

5.4.2. Optimisation of gel entrapment

Heating nisin at 121 °C and 118 °C for 15 min resulted in a 15.9% and 11.7% loss of nisin respectively, whereas there was only 1.59% (± 0.04, n = 3) loss of nisin detected in the products when heated at 115 °C for 15 min in a pH 3 solution. This low loss at 115 °C is in agreement with published work (Davies et al., 1998).

A HACS gel comprising 43% (w/w) HACS and 2% (w/w) enriched nisin was produced by heating at 115 °C for 15 min, retrograding at 4 °C and broken up using a mincer. Analysing the entrapment efficiency of this gel showed only 49.7% (± 1.8, n = 3) of the original nisin was unbound. Therefore these parameters became the standard process conditions and blend for subsequent HACS gel preparations. Light microscopy showed that raw HACS granules (Fig. 5.2A) if heated at 115 °C for 15

min, achieve partial gelatinisation with the granules swelling and losing their extinction cross, once cooled these granules are bound together in a retrograded HACS gel (Fig. 5.2B).

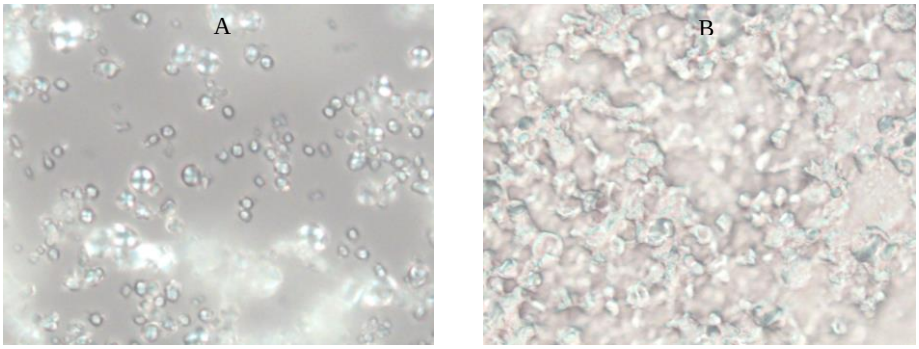

Fig. 5.2. Raw HACS granules (A) and the gel produced by heating HACS at 115 °C for 15 min and subsequently retrograded at 4 °C for16 h (B). Viewed by bright field at 20X magnification.

5.4.3. Bacterial fermentation

In order to demonstrate that nisin is retained within the HACS gel after digestion a method to detect the release of nisin from the gel was required which would not impact on nisin activity. As enzymatic approaches had been ineffective in Chapter 4, a bacterial fermentation approach was pursued. *R. bromii* was chosen as it has been identified as keystone species in resistant starch fermentation (Ze et al., 2012).

A cell free supernatant from a stationary phase culture of *R. bromii* had negligible ability to ferment HACS indicating that live cells are required for fermentation (Fig. 5.3). As the presence of starch is known to induce the production of α-amylase in microbes (Gupta et al., 2003) this result is unsurprising.

A stationary phase *R. bromii* culture fermented more HACS gel than a freshly seeded culture (Fig. 5.3). While having the capability to ferment resistant starch, *R. bromii* can also grow on simple carbohydrates such as glucose, galactose

and fructose (Mukhopadhya et al., 2018) and bacteria often show a hierarchical preference for carbohydrate fermentation (Tuncil et al., 2017). It is likely that *R. bromii* does not ferment the HACS until other carbohydrate sources in the media including those from the rumen fluid have been metabolised, this could explain why the stationary phase culture digested more HACS than freshly inoculated culture. However, *in vivo*, as simple sugars are absorbed higher up in the GIT system it is likely that the concentration of mono and disaccharides in the colon would be low giving *R. bromii* a competitive advantage in the lower GIT, given its ability to ferment resistant starch.

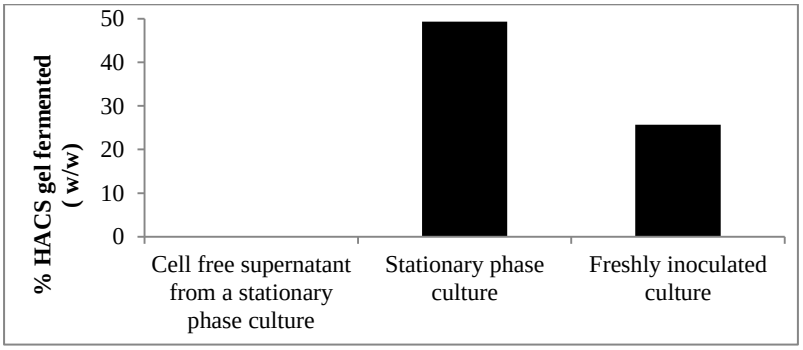

Fig. 5.3. Effect of growth stage and presence of *R. bromii* on amount of HACS gel fermented. Fermentations comprised 0.5 g of HACS gel and 7.5 mL of either freshly seeded culture, stationary phase culture or cell free supernatant from a stationary phase culture, which were incubated at 37 °C for 22 h under anaerobic conditions. The % w/w HACS fermented was calculated with respect to uninoculated controls.

To increase the amount of HACS fermented, the relationship between the starting amount of HACS and the proportion and total mass of HACS fermented was investigated. The greater the original mass of HACS in a sample, the greater the mass of HACS fermented but the lower the proportion of the sample that is fermented (Fig. 5.4).

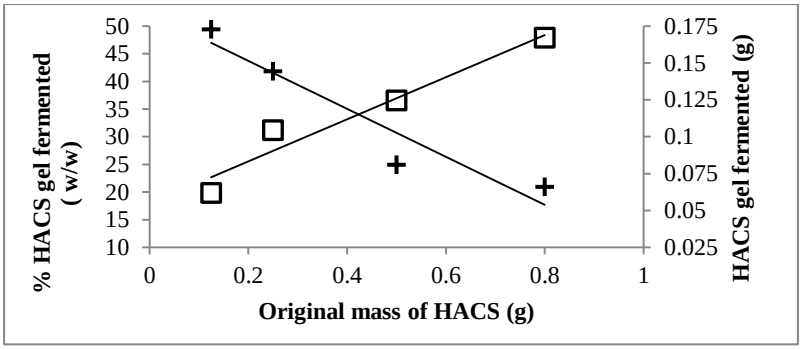

Fig. 5.4. Effect of substrate concentration on the amount of HACS gel fermented by *R. bromii*. The % w/w and mass of the HACS gel fermented are indicated by + and □ respectively. Fermentations comprised 7.5 mL of stationary phase cultures of *R. bromii* and 0.125, 0.25, 0.5 or 0.8 g of HACS gel and were incubated at 37 °C for 22 h under anaerobic conditions. The % w/w HACS fermented was calculated with respect to the uninoculated controls.

The starch fermentation preferences of colonic bacteria can depend on the molecular size of the starch and they can also have a preference for amylose or amylopectin (Tuncil et al., 2017). This sequential fermentation of starch components could explain how the increase in substrate results in an increase in mass fermented but a reduction in the total % w/w fermented. It is noted that the slope of the % gel fermented line implies that further reductions in the substrate would not increase the % fermented significantly above 50% w/w.

Using a bioreactor, with anaerobic conditions maintained by continuous nitrogen sparging and pH maintained at 6.8, did not cause a noticeable increase in the amount of HACS fermented and the stationary phase cultures still gave greater digestion of retrograded HACS gel (Fig. 5.5).

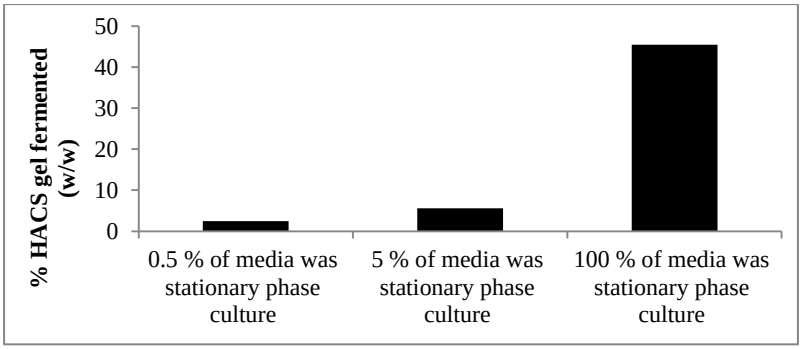

Fig. 5.5. Effect of anaerobic fermentation at 37 °C for 20 h in bioreactors with pH control and continuous nitrogen sparging on the amount of HACS gel fermented by *R. bromii*. Fermentations comprised .5 g of HACS gel and 200 mL of *R. bromii* culture. The % w/w HACS fermented was calculated with respect to the uninoculated controls.

Further attempts to increase the amount of retrograded HACS gel fermented, such as longer fermentation periods of up to 96 h did not result in greater than 50% of the retrograded HACS being fermented. However, this is comparable to (Ze et al., 2012) who achieved 43.1% fermentation of retrograde HACS using *R. bromii*.

MALDI TOF MS and RP-HPLC was used to detect and quantify the nisin released during the fermentation of HACS containing nisin by *R. bromii*. Although nisin was detected in the media by MALDI TOF MS the concentration was insufficient for quantification by RP-HPLC. In controls without HACS or bacteria, nisin was detectable by HPLC in the media, therefore it was deduced that the fermentation of HACS containing nisin by *R. bromii* did not release the majority of nisin from the gels.

5.4.4. Simulated chewing

To look at the importance of particle size in determining nisin release from HACS gels simulated digestions (by the second approach described in section 5.2.7) were performed on samples with particles sizes of 0.8 × 0.5 ×0.5 cm and 1.7 × 1.6

× 1.5 cm. The solution was tested by RP-HPLC to quantify the released nisin. The greatest nisin was detected after gastric phase and was 40% and 19% respectively.

As the particle size of the HACS gels had a major effect on the retention of the entrapped nisin during digestion, to accurately model the chewing of nisin a 'chew and spit' (Wickham et al., 2009) approach was employed. Modelling chewing time using a single 1 g gel block allowed more consistent chewing times than using smaller particles with the same total mass. A 1 g gel had been prepared with 45% HACS (w/w) time took 7.1 (± 0.3, n = 4) s to chew to the point of swallowing. Further blocks were chewed for this time and images were taken of the products which were measured by image analyses software (Fig. 5.6). It was determined that for 1 g gel blocks prepared with 45% HACS (w/w), the mean diameter after chewing was 1.8 (± 0.1, n = 3) mm. Subsequently, the simulated chewing phase comprised manually chopping the gels into particles of 1.8 (± 0.2) mm in diameter.

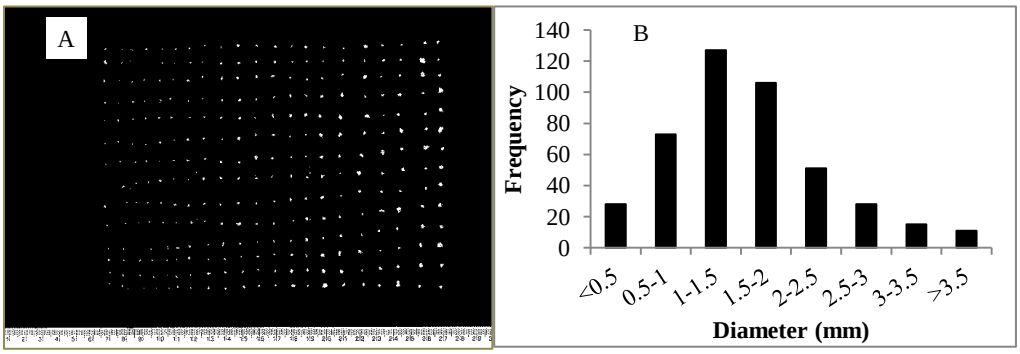

Fig. 5.6. Measurement of particle size of products of chewing. The particles (A) were measured using the Image J software (version 1.48) (Schneider et al., 2012) to obtain their equivalent spherical diameter (B).

5.4.5. Simulated digestion

Simulated digestion comprised an oral phase, gastric and intestinal stage. So that the nisin released from the HACS gel could be quantified it was necessary to perform it without proteases or bile. There were two approaches as seen in Table 5.1.

The first approach (approach A) used the native pHs and α-amylase to digest the HACS, whereas the second approach (approach B) was to account for the effect of pH on nisin and thus all stages were at pH 3 as this is where nisin is most stable (Davies et al., 1998) and as porcine pancreatic α-amylase is denatured at pH 3 (Gopal & Muralikrishna, 2009) this was not included.

Table 5.1. Simulated digestion approaches.

Phases	Approach A	Approach B
Oral phase (2 min)	pH 7, salivary α-amylase	pH 3
Gastric phase (2 h)	pH 3	pH 3
Small intestinal phase (2 h)	pH 7, pancreatic α-amylase	pH 3

All phases included phase appropriate minerals/electrolytes and were at 37 °C.

The test samples were nisin entrapped in HACS gel and the controls comprised nisin without HACS, both of which had undergone the same processing including the heat treatment. In approach A (Fig 5.7A) there was less nisin detected in solution for the test sample than the control samples after each digestion phase. This implies that the HACS gels (test samples) are retaining a portion of the original nisin after digestion.

However, the amount of nisin detected in solution from both the test and control samples is lower in the intestinal phase than the gastric phase. This is likely due to the nisin degrading at pH 7 and 37 °C as nisin is unstable above pH 6 with a temperature dependent decomposition rate (Kelly, Reuben, Rhoades, & Roller, 2000).

To test if nisin was being degraded by the neutral pH, a second digestion approach (approach B) was implemented. This confirmed the nisin loss was due to the neutral pH, although there was still less nisin detected in solution for the test samples than the control samples (Fig. 5.7B).

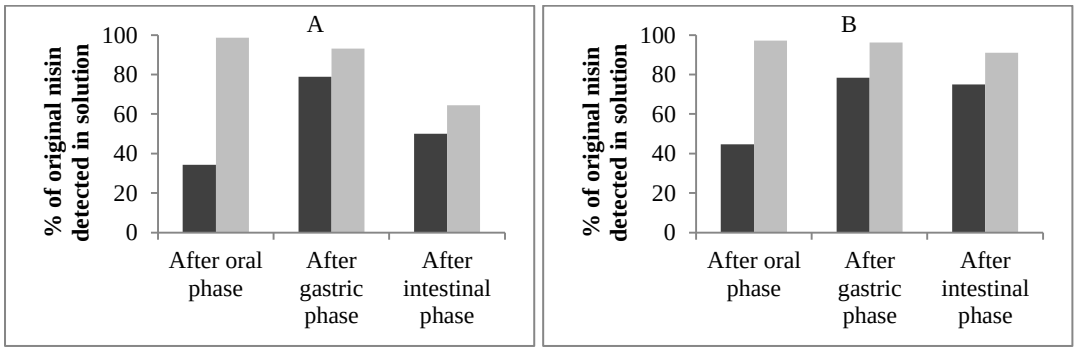

Fig. 5.7. Simulated digestions of nisin entrapped in HACS gel (test samples) (black bars) and nisin without HACS (control samples) (grey bars). All samples are comprised of 2% enriched nisin (w/w). The samples were subject to a simulated digestion with α-amylase and native pH (A) and without α-amylase and all phases at pH 3 (B). Samples were taken after the oral, gastric and intestinal phase had their nisin quantified by RP-HPLC.

The amount of nisin released during *in vitro* digestion could not be accurately quantified as nisin is unstable at the pH of the small intestine (pH 7) (Kelly et al., 2000) and while nisin is stable at pH 3 (Davies et al., 1998), porcine pancreatic α-amylase is inactivated at that pH (Gopal & Muralikrishna, 2009).

There are many proteinaceous bioactives that have high thermal stability, including the bacterially produced antibacterial peptides that are traditionally classified as class II bacteriocins (Casteels et al., 1989; Collado et al., 2005; Deraz et al., 2005; Klaenhammer, 1993; Oh et al., 2000; Singh & Vij, 2018). Therefore the approach taken here using nisin as a prototype peptide could be applied to other bioactive peptides molecule whose target site is the colon.

5.5. Conclusions

Nisin can be entrapped in a HACS gel by gelatinising and retrograding the HACS in the presence of nisin and nisin is resistant to the heat treatment required to generate such a gel. The size of the HACS gel particles affect release of nisin in simulated digestions and it was necessary to apply a chewing model so subsequent

release during simulated digestion could be accurately modelled. It appears that a portion of the nisin remains entrapped in the HACS gel after a simulated digestion, however due to the pH at the small intestine phase (pH 7) degrading the nisin as it is released, the amount of nisin remaining within the HACS gel cannot be quantified with certainty. To determine the nisin entrapped in the HACS gel and as previous enzymatic and chemical methods had been unsuccessful, a fermentation method based on the starch digesting bacteria *R. bromii* was developed. It was deduced that *R. bromii* only ferments HACS gel when other nutrient sources are exhausted and the presence of the HACS gel is required to induce production of the necessary amylases. Although greater proportion of the HACS was fermented than in previously published results, it was insufficient to release the majority of the bound nisin.

Therefore it does not seem possible to conclusively demonstrate the efficiency of this delivery system using *in vitro* models and an *in vivo* model would be required to prove the suitability of this system for bioactive delivery to the colon. Therefore an *in vivo* trial in mice was undertaken to determine the ability of HACS to deliver biologically active nisin to the lower GI tract (Chapter 7).

5.6. References

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Chapter 6

Fluorescent labelling of nisin to determine its localisation in starch gels

I would like to acknowledge the assistance of Mr. Carlos Rial with the fluorescent labelling, purification of the labelled peptides and spectrophotometric analyses. I would like to acknowledge the assistance of Dr. Deirdre Kennedy with the confocal microscopy.

6.1. Abstract

Nisin is an antimicrobial peptide showing activity against a broad range of Gram positive bacteria and is widely used as a food preservative. Fluorescent labelling of nisin would allow the determination of its localisation and release from a product. In the context of oral delivery systems for bioactive peptides, fluorescent labelling allows determination of the efficiency of the entrapment, the degree of protection and the rate of release. A system for orally delivering bioactive peptides to the colon by entrapping them in high amylose corn starch (HACS) gels was previously developed using nisin as a model peptide. The entrapment procedure required heating to 115 °C for 15 minutes at pH 3. Due to the detrimental effect of these processing conditions fluorescein isothiocyanate (FITC) was found to be unsuitable as a label. The Alexa Fluor® range of fluorescent labels have greater stability than conventional labels. Using an anion exchange approach allowed up-scaling of a previously reported approach for labelling nisin with and Alexa Fluor® 647. This label was able to remain conjugated to the nisin and maintain its fluorescent properties after processing. Although Alexa Fluor® 647 has been previously shown to bind to nisin without affecting its antibacterial activity, in this study nisin lost its antibacterial activity when bound to Alexa Fluor® 647. Starch gels in which nisin labelled with Alexa Fluor® 647 was entrapped, were examined by confocal microscopy and the labelled nisin appeared to localise at the surface of the starch granules.

6.2. Introduction

To avail of the health benefits of ingestion of bioactive peptides such as insulin or calcitonin, they require a delivery system to protect them from digestion, release them at the target site and enable their absorption. The distribution of the bioactive peptides in the matrix of the delivery system affects both the degree of protection from digestion and the rate of release; thus the efficiency of the system. To determine the distribution of the bioactive peptides in the matrix of the delivery system and also their release from the matrix, bioactive peptides are often fluorescently labelled.

Fluorescent labels that have been used in previous studies include 5-(6)-carboxytetramethylrhodamine succinimidylester, which was used to label salmon calcitonin that was entrapped in a poly(lactic acid) (PLA) matrix (Brunner, Minamitake, & Gopferich, 1998), fluorescein isothiocyanate (FITC), which was used to label Lysine-Arginine-Phenylalanine-Lysine that was entrapped in a calcium-alginate matrix (Hurteaux, Edwards-Levy, Laurent-Maquin, & Levy, 2005) and Alexa Fluor® 488, which was used to label insulin that was entrapped in an alginate–chitosan matrix (Zhang et al., 2011).

Fluorescent labelling of proteins and peptides is achieved through both genetic approaches and through direct labelling of the native protein. Genetic approaches include incorporation of a fluorescent amino-acid sequence fused to the protein of interest and incorporation of a genetic encoded tag that can be complexed with a fluorochrome, while the primary approaches for direct labelling of the native proteins and peptides are antibody based systems (immunolabeling), organic dyes and quantum dots, which are a recently developed system (Giepmans, Adams,

Ellisman, & Tsien, 2006). The fluorophore-antibody complexes are typically 200 kDa (Giepmans et al., 2006) and a quantum dot and its linker proteins are comparable in size to a protein of 500-750 kDa (Jaiswal & Simon, 2004); this makes them likely to interfere with the functionality of any peptide bound to them. Due to the small size of organic dyes (for example FITC is 389 Da (Wischke & Borchert, 2006)), they are less likely to interfere with the activity of a peptide (Resch-Genger et al., 2008).

Nisin is a bioactive peptide that has activity against a wide range of Gram positive bacteria and is widely used as a food preservative (Abee & Delves-Broughton, 2003). When taken orally nisin is digested during gastrointestinal transit (Younes et al., 2017). Nisin is highly heat stable and when at pH 3 it can be heated to 115 °C for 20 min with <5% loss in activity (Davies et al., 1998). In Chapter 5 nisin was entrapped in a high amylose corn starch (HACS) gel matrix through a heat treatment of 115 °C for 15 minutes at pH 3, in order to enable its colonic delivery.

Fluorescent labelling of nisin would allow its localisation in the HACS matrix to be determined. There are two considerations that inform the choice of label for the fluorescent labelling of nisin. Firstly the label must not interfere with the activity of the nisin; to do this it must be small enough not to cause steric interference and it must be bound on the C-terminal; the addition of a label to other locations, particularly the N–terminal, is known to inhibit nisin activity (Guiotto et al., 2003; Slootweg et al., 2013). Secondly the label must be able to maintain its bond with nisin and not loose fluorescence under the pH and temperature conditions required to entrap nisin in a HACS gel.

The Alexa Fluor® range of fluorescent labels have significantly greater pH and heat stability than conventional fluorescent labels (Kapoor et al., 2009; Panchuk-

Voloshina et al., 1999). Alexa Fluor® 647 has been covalently bound to the C-terminal of nisin and there was no loss in nisin activity as determined by carboxyfluorescein leakage assay (Scherer et al., 2013).

In this chapter nisin was fluorescently labelled to determine its localisation when entrapped in a HACS gel. Initially labelling was attempted using conventional fluorescent label FITC to confirm that conventional fluorescent labels were insufficient for the requirements. Subsequently nisin was labelled using Alexa Fluor® 647 based on the method of Scherer et al. (2013). The major modification to the method of Scherer et al. (2013) was the use of an ion exchange chromatography procedure for purification of the product of the labelling reaction, this allowed the upscaling of the labelling procedure.

6.3. Methods

6.3.1. Preparation of nisin

Nisin was enriched from a commercial nisin preparation (Nisaplin®, DuPont, Beaminster, UK) by salting out as described in Chapter 2 (Gough et al., 2017). This is referred to in the text as enriched nisin.

To further purify the enriched nisin by removing non-nisin peptides, a HiTrap™ CM fast flow (FF) cation exchange column was used (17-6002-33, VWR, Dublin, Ireland). The cation exchange column had a column volume of 1 mL and a flow rate of 1 mL/min was used. The column was first washed with 5 column volumes of 1 M NaCl, 0.1M MES buffer pH 6. Then it was equilibrated with 5 column volumes of 0.1 M MES buffer pH 6. Ten mL of a 2 mg/mL enriched nisin in

0.1M MES buffer pH 6 was loaded on to the column. The column was washed with 10 column volumes of 0.1M MES buffer pH 6 to remove unbound material including non-nisin peptides. The column was then washed with 20 column volumes of 1 M NaCl, 0.1M MES buffer pH 6 to elute the nisin. To remove the salt from the purified nisin, it was precipitated with 20% (v/v) trichloroacetic acid (TCA) overnight at 4 °C. To remove the TCA, the purified nisin was washed twice with 4 °C acetone. The nisin was quantified by reversed-phase high performance liquid chromatography (RP-HPLC) as described below in section 6.3.6.

6.3.2. Labelling

Labelling of enriched nisin with fluorescein isothiocyanate (FITC) (F7250, Sigma Aldrich, Arklow, Ireland) was performed as per manufacturer's instructions. The labelled samples were brought to a final FITC concentration of 13 µg/mL in both a 0.1 M sodium carbonate pH 9 buffer and a 0.1 M citric acid buffer pH 3.

Enriched nisin and nisin purified by cation exchange were labelled with Alexa Fluor® 647 Hydrazide (A20502, Fisher Scientific, Dublin, Ireland) using the method of Scherer et al. (2013) with some modifications. For each labelling reaction 46 µL of Alexa Fluor® 647 Hydrazide in DMSO (16.5 µg/µL) was added to 0.5 mL of nisin in 0.1 M MES pH 5 buffer (5 mg/mL), which corresponds to a 0.8 mol dye to 1 mol peptide ratio. Immediately before use a solution of 500 mM *N*-(3-Dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC) in 0.1 M MES buffer was prepared and 14 µL of this added to the reaction mixture. This was incubated overnight at room temperature.

To purify the labelled nisin a HiTrap™ Q Sepharose FF anion exchange column was used (17-5156-01, VWR, Dublin, Ireland). The anion exchange column had a column volume of 5 mL and a flow rate of 5 mL/min was used. The column was first washed with 5 column volumes of 1 M NaCl, 100 mM bis-tris propane buffer pH 9 and then it was equilibrated with 5 column volumes of 100 mM bis-tris propane buffer pH 9. The products of a single labelling reaction were diluted in 100 mM bis-tris propane pH 9 buffer to a final volume of 10 mL and loaded on the column. The column was washed with 10 column volumes (50 mL) of 100 mM bis-tris propane pH 9. The column was then washed with 10 column volumes of 100 mM bis-tris propane pH 7 to elute the Alexa Fluor® 647 conjugated nisin. Finally the column was washed with 10 column volumes of 1 M NaCl 100 mM bis-tris propane pH 9 buffer.

The elution product (Alexa Fluor® 647 conjugated nisin) was condensed by freeze drying and then desalted using a Vivaspin® 15R ultrafiltration spin columns with a 2 kDa molecular weight cut off (MWCO) (VS15RH91, Sartorius, Dublin, Ireland), according to the manufacturer's instructions. The reduction of salt was monitored by conductivity using a MultiLine® P3 conductivity meter (WTW, Weilheim, Germany). The products of desalting were freeze dried.

6.3.3. Matrix-assisted laser desorption/ionization time of flight mass spectroscopy (MALDI TOF MS)

The nisin bound to Alexa Fluor® 647 was analysed by MALDI TOF MS using an Axima TOF^2 (Shimadzu Biotech, Kyoto, Japan) as previously described

(Field et al., 2012) to confirm the conjugation had occurred and that a single fluorescent label was applied per peptide.

6.3.4. Heat treatments

Heat treatments were performed at 115 °C for 15 minutes to match the production conditions for entrapment of nisin in the HACS gel matrix as described in Chapter 5.

6.3.5. Spectrophotometry

Fluorescencent and UV/Visible spectrophotometry were performed using a Varian Cary Eclipse and a Varian Cary 1 (Agilent Technologies, Little Island, Ireland) and a Jenway 6300 (Cole-Parmer, Stone, UK). The quantification of the Alexa Fluor® 647 conjugated nisin was performed by absorbance at 650 nm (excitation maximum of Alexa Fluor® 647 (Anderson & Nerurkar, 2002)) with respect to its molar extinction coefficient and to a standard curve produced from Alexa Fluor® 647 of known concentrations.

6.3.6. Reversed-phase high performance liquid chromatography

Free nisin, free Alexa Fluor® 647 and nisin bound to Alexa Fluor® 647 were detected by using reversed-phase high performance liquid chromatography (RP-HPLC) based on published methods (Buonocore et al., 2003; Chollet, Sebti, Martial-Gros, & Degraeve, 2008). RP-HPLC was carried out using a Waters e2695

separation module with a Waters 2489 UV/visible detector, running on Waters Empower software (Waters, Dublin, Ireland) and a Jupiter 5 µm, C18, 300A, 250 mm × 4.6 mm from Phenomenex (Macclesfield, UK). Solvent A was 0.1 % (v/v) trifluoroacetic acid (TFA) (Sigma-Aldrich, Arklow, Ireland) in Milli-Q® water (Merck Millipore, Carrigtwohill, Ireland), and solvent B was 90 % (v/v) HPLC-grade acetonitrile (Thermo Fisher Scientific, Dublin, Ireland) containing 0.1 % TFA (v/v) in Milli-Q® water. A linear gradient from 0% B to 55.6% B over 25 min was run at a flow rate of 1.0 mL/min. Nisin was detected by absorbance at 214 nm and Alexa Fluor® 647 and Alexa Fluor® 647 conjugated to nisin were detected at 650 nm.

6.3.7. Activity assay

The biological activity of nisin bound to Alexa Fluor® 647 and unbound nisin were compared by agar diffusion activity assay (Ryan, Rea, Hill, & Ross, 1996). *L. lactis* subsp. *cremoris* HP, the indicator strain was grown overnight in M17 broth (Oxoid, Basingstoke, UK) containing 0.5% lactose (VWR, Dublin, Ireland) (LM17). LM17 agar was tempered to 45 °C and seeded with 0.5 % of the indicator strain. Twenty millilitre aliquots of the seeded agar were dispensed into sterile petri dishes, these were allowed to solidify and wells of 5 mm in diameter were bored in the agar. Serial two-fold dilutions of the samples were dispensed into the wells in 50 µL aliquots and the plates were incubated overnight at 30 °C. The activity of the nisin resulted in zones of inhibition surrounding the wells.

6.3.8. Entrapment of nisin in starch gel

Starch gels with nisin were composed of 55% (w/w) dilute HCl, 44% (w/w) HACS and 1% (w/w) enriched/labelled nisin (3% (w/w) of nisin was labelled with Alexa Fluor® 647), with a final pH of 3. The gels were heated to 115 °C for 15 min and subsequently incubated at 4 °C for a minimum of 16 h to ensure thorough retrogradation.

6.3.9. Confocal microscopy

Confocal microscopy was performed using a Leica TCS SP Confocal Laser Scanning Microscope (Leica Microsystems, Heidelberg GmbH, Mannheim, Germany). Starch gels containing entrapped nisin of which 3% (w/w) was labelled with Alexa Fluor® 647, were viewed using a 10× air and 63× oil immersion lens with zooms of 1×, 3× and 5×. The labelled nisin was excited with a He–Ne laser with a wavelength of 633 nm and the detector was set in the range of 656 to 682 nm. The images of the fluorescence were overlaid with images of the gel taken using differential interference contrast (DIC).

6.4. Results and discussion

An examination of the pH and heat stability of FITC conjugated to nisin is shown in Fig. 6.1A, B and C. FITC has optimum activity at pH 9 (Sherr, Caron, & Sherr, 1993) and has an absorbance maximum at 495 nm and an emission maximum at 519 nm (Hermanson, 2013). In Fig. 6.1A free FITC at pH 9 had an absorbance

maximum at 495 nm; however conjugation to nisin induced a blue shift to an absorbance maximum of 418. When this nisin conjugated FITC underwent a heat treatment at pH 9 (Fig. 6.1A) the absorbance maximum reverted to that of free FITC; this may be due to the heat treatment releasing the FITC from the nisin.

Examination of the absorbance of FITC conjugated to nisin before and after heat treatment at pH 3 and free FITC at pH 3 showed they all had an absorbance maximum at 435 nm (Fig. 6.1B). This implies that the pH of 3, blue shifted the absorbance from the normal absorbance maximum of 495 nm.

With respect to fluorescence after heat treatment at pH 3, FITC conjugated to nisin had greater fluorescence at 435 nm (the pH 3 absorbance maximum) than 495 nm (the normal excitation maximum (Er, 2006) (Fig. 6.1C). Irrespective of this, FITC conjugated to nisin and heat treated at pH 3 had distinctly lower fluorescence than the control (FITC conjugated to nisin at pH 9 and excited at its absorbance maximum of 418 nm) (Fig. 6.1C). FITC does not appear to be suitable for labeling nisin that will undergo the HACS gel encapsulation process (115 °C for 15 minutes at pH 3), due to its level of pH and temperature stability.

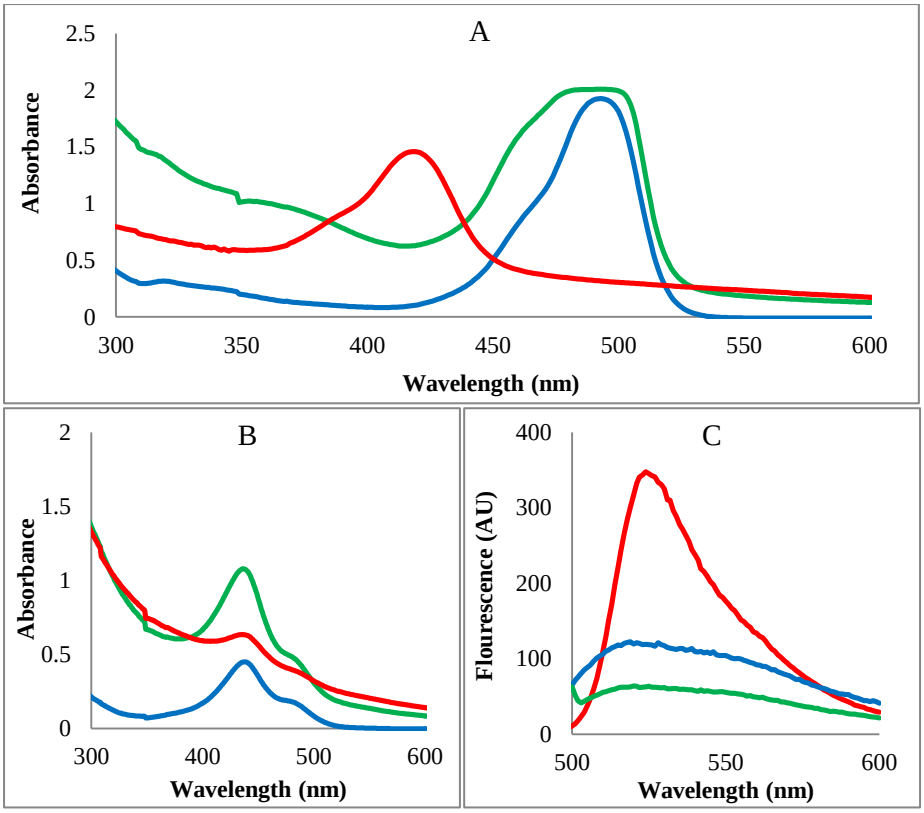

Fig. 6.1. Analysis of FITC conjugated to nisin. (A) overlay of absorbance of FITC conjugated to nisin before (—) and after (—) heat treatment at pH9 and free FITC at pH9 (—). (B) overlay of absorbance of FITC conjugated to nisin before (—) and after (—) heat treatment at pH3 and free FITC at pH3 (—). (C) overlay of fluorescence at of FITC conjugated to nisin at pH9 and an excitation of 418 nm (—) and after heat treatment at pH3 and an excitation of 435 nm (—) and 495 nm (—). Fluorescence is described in terms of arbitrary units (AU).

The Alexa Fluor® range of fluorescent labels have been demonstrated to have much greater temperature (Kapoor et al., 2009) and pH (Panchuk-Voloshina et al., 1999) stability than conventional fluorescent labels such as FITC. Alexa Fluor® 647 was chosen because it has been used in a published method to label nisin without affecting its antibacterial activity (Scherer et al., 2013).

Initially there was a concern that the Alexa Fluor® dye would label the non-nisin peptides in the enriched nisin and to avoid this problem the enriched nisin was further purified by cation exchange. However the same RP-HPLC profile resulted from labelling the enriched nisin and the cation exchange purified nisin. Therefore as labelling of non-nisin peptides in the enriched nisin was not an issue, for subsequent labelling reactions the enriched nisin was used without further purification.

Unlike FITC, there was no change in the absorbance spectrum of Alexa Fluor® 647 after conjugation to nisin, with the absorption maximum remaining at 650 nm. Due to the quantity of nisin used in the HACS gel entrapment protocol (Chapter 5), it was necessary to upscale the Scherer et al. (2013) method to have sufficient labelled nisin. Therefore in this study the products of the labelling reaction were purified using a scalable ion-exchange approach as opposed to the RP-HPLC approach used in Scherer et al. (2013).

To purify the product of the labelling, initially cation exchange (SP and CM Sepharose) was used. However, the nisin conjugated to Alexa Fluor® 647 did not bind to the columns even when they were run at a pH of 4 (4 is the lowest pH that Alexa Fluor® 647 is reported to be stable at (Thermo Fisher Scientific, 2018)). Because of this anion exchange approaches were tried and it was found that at pH 9 the nisin conjugated to Alexa Fluor® 647 bound to Q Sepharose.

Using ion-exchange to purify the labelling product allowed >25 times the Alexa Fluor® 647 conjugated nisin to be purified per run cycle, than was possible using the RP-HPLC approach described by Scherer et al. (2013).

The elution product (Alexa Fluor® 647 conjugated to nisin) of the anion exchange column gave a single distinctive peak when analysed by RP-HPLC and this peak is different to the free nisin or Alexa Fluor® 647 controls (Fig. 6.2). This shows the purification was successful, however it also shows that the hydrophobicity of the Alexa Fluor® 647 conjugated to nisin is distinct from that of nisin, which may impact on the antibacterial activity of the nisin.

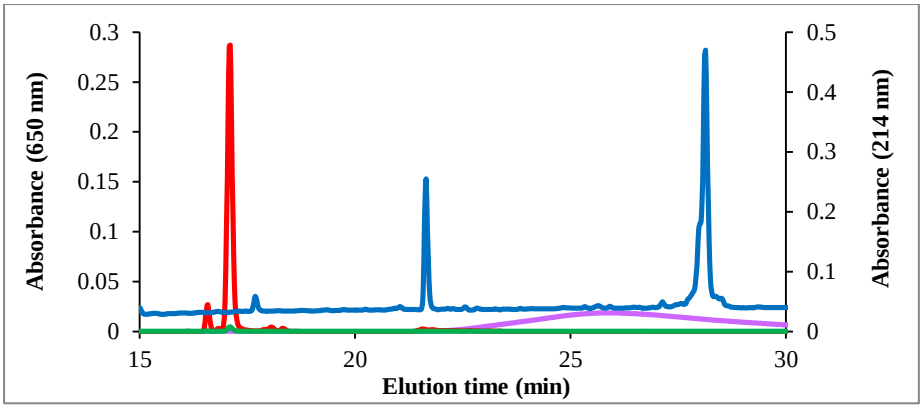

Fig. 6.2. Overlay of RP-HPLC chromatograms of the product of purification by anion exchange column and controls. Absorbance of Alexa Fluor® 647 conjugated to nisin after purification by anion exchange at 650 nm (—) and 214 nm (—), absorbance of Nisaplin® (nisin control) at 214 nm (—) and absorbance of Alexa Fluor® 647 (control) at 650 nm (—).

The Alexa Fluor® 647 conjugated to nisin was not detected by MALDI TOF MS. This was also reported by Esteban et al. (2011) who were also unable to detect Alexa Fluor® 647 peptide conjugates by MALDI TOF MS, but were able to detect Alexa Fluor® 488 peptide conjugates by the same approach. It is possible that the conjugates are insufficiently protonated due to the negative charges brought by the Alexa Fluor® 647 label (Sobek, Aquino, & Schlapbach, 2011).

Nisin has maximum stability during heat treatment at pH 3 (Davies et al., 1998) whereas the lowest reported pH that Alexa Fluor® 647 is stable at is pH 4 (Thermo Fisher Scientific, 2018). Comparing Alexa Fluor® 647 conjugated nisin that underwent the heat treatment at pH 3 to untreated Alexa Fluor® 647 conjugated nisin at pH4 by RP-HPLC showed that their absorbance was unaffected by processing conditions and that they remained conjugated (Fig. 6.3A), while comparing their fluorescence showed that this was also unaffected by the processing conditions (Fig. 6.3B).

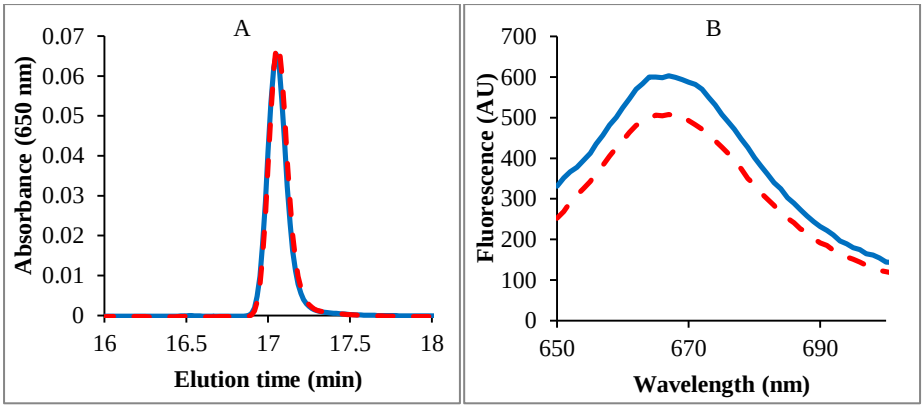

Fig. 6.3. Effect of heat treatment (115 °C for 15 min) at pH 3 on Alexa Fluor® 647 conjugated to nisin (– – –) compared to unheated controls at pH 4 (——). (A) overlay of RP-HPLC chromatograms with absorbance at 650 nm. (B) overlay of fluorescence spectrums using an excitation wavelength of 649 nm. Fluorescence is described in terms of arbitrary units (AU).

There was no antibacterial activity detected by agar well diffusion activity assay of nisin conjugated to Alexa Fluor® 647 despite it having a much greater nisin concentration than the positive control (Nisaplin®) (Fig. 6.4). Nisin conjugated Alexa Fluor® 647 was previously deemed to be biologically active due to its capacity to release carboxyfluorescein from liposomes composed of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) with 0.2 mole-percentage lipid II (Scherer et al., 2013); as the agar well diffusion activity assay used in this study tests nisin activity against live bacteria it can be considered a more accurate assessment of nisin activity. It is possible that the negative charge of the Alexa Fluor® 647 (-3, (Sobek et al., 2011)) reduced the overall charge of the conjugate too much for the nisin to be effective.

As the nisin component of the conjugate has lost its activity, presumably due to a change in overall charge, the behaviour and localisation of the conjugate in the HACS gel may not be representative of native nisin.

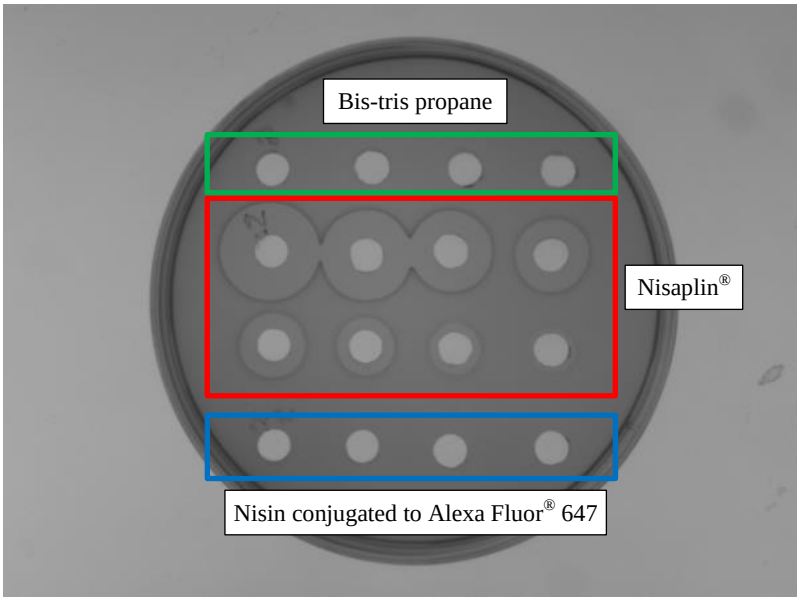

Fig. 6.4. Agar well diffusion activity assay. The first row (green box) is bis-tris propane at pH 7 and has a concentration of 100 mM in the first well. The second and third rows (red box) are Nisaplin® with the first well in the second row having a nisin concentration of 0.02 µg/µL and this dilution series continues into the third row. The fourth row (blue box) is nisin conjugated to Alexa Fluor® 647 and has a nisin concentration of 0.4 µg/µL in the first well.

Starch gels containing entrapped nisin of which 3% (w/w) was labelled with Alexa Fluor® 647, were analysed by confocal microscopy (Fig. 6.5A and B). The labelled nisin (red fluorescence) appeared to localise at the surface of the starch granules, however it is unclear if it was penetrating into the granule surface. As the fluorescence follows the curve of the granular surfaces, it is possible the labelled nisin was interacting with the granular surfaces.

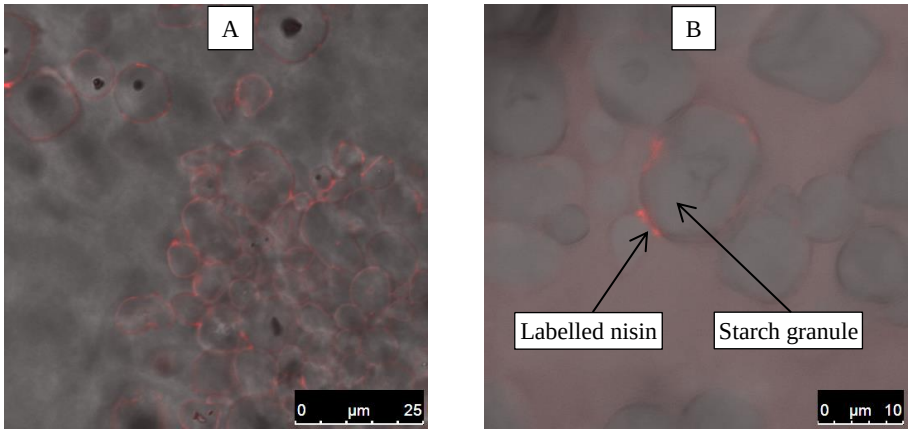

Fig. 6.5. Confocal microscopy of starch gels containing entrapped nisin of which 3% (w/w) was labelled with Alexa Fluor® 647. Images were obtained using a 63× oil immersion lens with a zoom of 3× (image A) and 5× (image B). Images of fluorescence obtained by excitation at 633 nm and detection in the range of 656 to 682 nm were overlaid with images taken using differential interference contrast (DIC).

6.5. Conclusions

The fluorescent label AlexaFluor® 647 remained conjugated to the nisin and maintains its fluorescent properties after undergoing the processing conditions required for gel entrapment (pH 3, 115 °C, 15 minutes). However, the fluorescent label inhibited the antibacterial activity of the nisin. The labelled nisin appears to localise at the surface of the starch granules and may be interacting with them.

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Chapter 7

Oral delivery of nisin in resistant starch based matrices alters the gut microbiota in mice

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7.1. Abstract

There is a growing recognition of the role the gastrointestinal microbiota plays in health and disease. Ingested antimicrobial proteins and peptides have the potential to alter the gastrointestinal microbiota; particularly if protected from digestion. Nisin is an antimicrobial peptide that is used as a food preservative. This study examined the ability of nisin to affect the murine microbiota when fed to mice in two different starch based matrices; a starch dough comprising raw starch granules and a starch gel comprising starch that was gelatinized and retrograded. The effects of the two starch matrices by themselves on the microbiota were also examined. Following 16S rRNA compositional sequencing, beta diversity analysis highlighted a significant difference ($p = 0.001$, $n = 10$) in the murine microbiota between the four diet groups. The differences between the two nisin containing diets were mainly attributable to differences in the nisin release from the starch matrices while the differences between the carriers were mainly attributable to the type of resistant starch they possessed. Indeed, the differences in the relative abundance of several genera in the mice consuming the starch dough and starch gel diets, in particular *Akkermansia*, the relative abundance of which was 0.5% and 11.9%, respectively ($p = 0.0002$, $n = 10$), points to the potential value of resistance starch as a modulator of beneficial gut microbes. Intact nisin and nisin digestion products (in particular nisin fragment 22-31) were detected in the faeces and the nisin was biologically active. However, despite a threefold greater consumption of nisin in the group fed the nisin in starch dough diet, twice as much nisin was detected in the faeces of the group which consumed the nisin in starch gel diet. In addition, the relative abundance of

three times as many genera from the lower gastrointestinal tract (GIT) were significantly different ($p < 0.001$, $n = 10$) to the control for the group fed the nisin in starch gel diet, implying that the starch gel afforded a degree of protection from digestion to the nisin entrapped within it.

7.2. Introduction

The gastrointestinal microbiota impacts on the health of the host in variety of ways, including through its potential to protect against infection, provide nutrients and influence on bodyweight (Clarke et al., 2012; Jandhyala et al., 2015; Nicholson et al., 2012). The composition of the microbiota, and thus its health effects, can be altered by a variety of means, including antimicrobials and diet (Chung et al., 2016; Cotter, Stanton, Ross, & Hill, 2012; Martinez et al., 2010).

Nisin is an antimicrobial peptide with broad activity against Gram positive bacteria produced by strains of *Lactococcus lactis* subsp. *lactis* (Abee & Delves-Broughton, 2003). Nisin has been approved for use as a food preservative by both US Food and Drug Administration, (FDA) (US Food and Drug Administration, 1988) and by the European Food Safety Authority (EFSA) with its assigned E number being E 234 (Younes et al., 2017).

Nisin is very stable at low pH and at pH 3 there is <5% loss of activity when heated to 115 °C for 20 min (Davies et al., 1998). However, while relatively resistant to passage through the acidic conditions in the stomach, nisin can be digested by pancreatin in the small intestine (Gough, O'Connor, et al., 2017; Heinemann & Williams, 1966), primarily by its trypsin and chymotrypsin components and

therefore may not reach the lower gastrointestinal tract (GIT) in an intact form (Chan et al., 1996; Jarvis & Mahoney, 1969).

Few *in vivo* studies (Table 7.1) have investigated how dietary supplementation with nisin affects the microbiota of the lower GIT (Bernbom et al., 2006; Józefiak et al., 2013; Kieronczyk et al., 2016; Lauková et al., 2014) and no previous *in vivo* study has employed a high throughput sequencing (HTS)-based approach to examine the impact of nisin on the entire microbiota. Nisin has been consumed *in vivo* at up to 239 mg per kg body weight per day without any adverse effects on food consumption, body weight, haematology, ophthalmology or gross pathology (Hagiwara et al., 2010). Although nisin doses of up to 173.9 mg per kg body weigh per day had no impact on the microbiota in a study on rats (Bernbom et al., 2006), nisin has been seen to influence the microbiota in some way in the majority of *in vivo* studies including those on mice, chickens and rabbits and in *in vitro* bovine and human microbiota studies (Table 7.1). However, the variation in methods used, and the previous absence of detailed HTS-based investigations, make direct comparisons difficult (Table 7.1).

Table 7.1. Comparison of selected *in vivo* and *in vitro* models of nisin activity.

In vivo or *in vitro* model	Nisin delivery	Highest nisin consumption per kg body weight per day	Nisin in faeces	Microbiota			Rate of weight gain	Reference
				Location tested	Test method	Nisin effect		
Mice	Oral	400 mg	Not tested	Not tested	Not tested	Not tested	Increased in one test group, no change in all other test groups	Shtenberg and Ignatev (1970)
Mice	Oral	161 mg (starch dough diet), 54 mg (starch gel diet)	Yes	Faeces	16S rRNA MiSeq sequencing	Yes	No change	This paper

Mice	Oral	Not available	Not tested	Not tested	Not tested	Not tested	No change	de Pablo et al. (1999)
Mice	Intraperitoneal injection	Not available	Not tested	Faeces	Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rDNA	Yes	Not tested	van Staden, Brand, Endo, and Dicks (2011)
Rats	Oral	225 mg (males), 239 mg (females)	Not tested	Not tested	Not tested	Not tested	No change	Hagiwara et al. (2010)
Rats	Oral	174 mg *	Yes	Faeces	Plating on selective media and denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rDNA	No	Not tested	Bernbom et al. (2006)
Rats	Oral	50 mg	Not tested	Not tested	Not tested	Not tested	No change	Reddy, Gupta, and Aranha (2011)
Rats	Oral	50 mg	Not tested	Not tested	Not tested	Not tested	No change	Gupta, Aranha, and Reddy (2008)
Rats	Oral	10 mg	Not tested	Not tested	Not tested	Not tested	No change	Reddy, Aranha, Gupta, and Yedery (2004)
Quails	Oral	52 mg	Not tested	Not tested	Not tested	Not tested	No change	Ozdogan and Ustundag (2015)
Chickens	Oral	10 mg	Not tested	Ileum	DAPI staining and fluorescent *in situ* hybridization (FISH)	Yes	Increased	Józefiak et al. (2013)
Chickens	Oral	10 mg	Not tested	Ileum	DAPI staining and fluorescent *in situ* hybridization (FISH)	Yes	Increased	Kieronczyk et al. (2016)
Rabbits	Oral	20 µg	Not tested	Faeces	Plating on selective media	Yes	Increased	Lauková et al. (2014)
Fermentation vessel (bovine rumen)	Not relevant	Not relevant	Not relevant	Not relevant	16S RNA MiSeq sequencing	Yes	Not relevant	Shen, Liu, Yu, and Zhu (2017)
Fermentation vessel (human colon)	Not relevant	Not relevant	Not relevant	Not relevant	q-PCR coupled to propidium monoazide treatment	Yes	Not relevant	Le Lay et al. (2015)

* Extrapolated based on standard (Lillie, Temple, & Florence, 1996) weight for rats of that age and breed.

Starch is the primary carbohydrate source in the adult western diet (Sibley, 2004). Starch is comprised of the carbohydrate polymers amylose and amylopectin, and in plants these are arranged into semi-crystalline starch granules, which are of 0.1 to 200 μm in diameter. When 'raw' starch granules are suspended in water and heated, the amylose and amylopectin disassociate, with the granules leaching amylose and absorbing water causing them to swell and ultimately dissipate. When the solution is subsequently cooled, the amylose and amylopectin re-associate, turning the solution into a starch gel, with the gel strength primarily determined by amylose content. These two stages are referred to as gelatinization and retrogradation (Alcázar-Alay & Meireles, 2015; Wang et al., 2015). Many types of food processing, including cooking, can cause starch to undergo gelatinization and retrogradation (Delcour et al., 2010) with co-present substances becoming entrapped in the resulting starch gel (Forssell, 2004).

The portion of starch that resists digestion in the small intestine is termed 'resistant starch' and varies between starch source and type. In the case of the type of starch used in this study (70% amylose starch from maize), the resistant starch content has been reported as 46% on a w/w basis (McCleary, McNally, & Rossiter, 2002). Starch that is resistant due to its granular nature is classified as type 2 resistant starch (RS2), whereas starch that is resistant due to retrogradation is classified as type 3 resistant starch (RS3) (Sajilata, Singhal, & Kulkarni, 2006). Due to the capacity of the resistant starch portion of a starch to resist digestion in the upper GIT and subsequently be fermented by colonic bacteria, starch based systems have been proposed for the colonic delivery of drugs and bioactive materials; these systems frequently use ethyl cellulose as a binder and are frequently produced through spray coating (Desai, 2005; Dimantov, Greenberg, Kesselman, & Shimoni,

2004; Freire et al., 2010; Milojevic et al., 1996; Pu et al., 2011; Recife, Meneguin, Cury, & Evangelista, 2017; Situ, Chen, Wang, & Li, 2014; Wilson & Basit, 2005).

The aim of this study was to determine the effect, *in vivo*, of orally consumed nisin on the lower GIT microbiota (as determined by 16S rRNA HTS of faecal samples (Suzuki & Nachman, 2016)) when nisin was incorporated into two different starch based matrices; a dough based on raw starch (RS2) and a gel based on starch that had undergone gelatinization and retrogradation (RS3). Additionally the potential of the starch matrices themselves to impact on the microbiota was examined.

7.3. Materials and methods

7.3.1. Reagents

High amylose corn starch (HACS) was obtained from Sigma Aldrich (S4180, Sigma Aldrich, Arklow, Ireland). Dextrose equivalent 12 maltodextrin (DE12 MD) was obtained from Roquette (Glucidex® 12, Roquette, Corby, UK). All other reagents were from Sigma Aldrich (Arklow, Ireland) unless otherwise specified.

7.3.2. Preparation of nisin

The nisin A preparation used in this study was Nisaplin® (DuPont, Beaminster, UK). This preparation was concentrated by salting out as previously

described (Gough, Gómez-Sala, et al., 2017). This resulted in a 57.7% nisin preparation which will subsequently be referred to in the text as enriched nisin.

7.3.3. Preparation of test diet pellets

Starch gels were prepared with and without nisin as follows. Starch gels with nisin were composed of 1% (w/w) enriched nisin, 44% (w/w) HACS and 55% (w/w) dilute HCl, with a final pH of 3. Starch gels without nisin were composed of 45% (w/w) HACS and 55% (w/w) dilute HCl, with a final pH of 3. The suspensions were split into 10 mL aliquots, heated at 115 °C for 15 min and subsequently incubated at 4 °C for a minimum of 16 h to ensure thorough retrogradation. Starch dough was prepared with and without nisin as follows. The starch dough balls with nisin comprised 1% (w/w) enriched nisin, 51.5% (w/w) HACS, 22.5% DE12 MD and 25% (w/w) dilute HCl. The starch dough balls without nisin contained 52.5% (w/w) HACS, 22.5% DE12 MD and 25% (w/w) dilute HCl. For the preparation of the nisin containing starch dough balls, the dilute HCL and enriched nisin (at pH 3) were heated at 115 °C for 15 min and allowed cool to room temperature before addition to the rest of the ingredients, to ensure that the treatment of the nisin in the starch dough was comparable with that of the nisin in the starch gel. All the components of the starch dough balls were then mixed in a laminar flow cabinet. Each starch dough ball was thoroughly kneaded to achieve homogeneity and firmness. The starch dough balls were stored at 4 °C until use.

7.3.4. Feeding schedule and sample collection

This study was carried out in accordance with European Directive 2010/63/EU. The protocol was approved by the University College Cork Animal Experimentation Ethics Committee (2011/005). Male C57BL/6JOlaHsd mice aged 3-4 weeks (Envigo, Alconbury, UK) were group housed (5 per cage) and were maintained in a 12:12 h light-dark cycle. During the initial 10 day acclimatization period, the mice were fed a standard nutritionally complete low-fat rodent diet (D12450B, Research Diets, New Brunswick, New Jersey, US); this diet is henceforth referred to as the nutritionally complete (NC) diet. Subsequently weight matched mice were assigned to receive the following test diets: starch dough (SD), starch dough containing nisin (SD-N), starch gel (SG) and starch gel containing nisin (SG-N) (n = 10 per test diet).

An overview of the feeding schedule is shown in Table 7.2. The feeding schedule involved initially switching the NC diets with the test diets for 2 h per day for three days and this was gradually increased to 8 h per day over the period of the trial as described in Table 7.2.The test diets were introduced gradually to acclimatize the animals to eating the starch based diets. The exposure to the NC diets thus decreased from 22 h to 16 h per day over the period of the trial. The test diets were replaced every 4 days to ensure the freshness of the diet pellets. As mice are nocturnal animals and the cage room was on a 12:12 h light-dark cycle, the food hoppers were switched to test diets at the beginning of the dark cycle (18:00). The food hoppers were weighed throughout the trial as described in Table 7.2 and additional food hoppers in empty cages were used as controls to measure the impact

of diet pellet drying on diet pellet weight. The hoppers were loaded with sufficient pellets of the test and NC diets to ensure that a sufficient quantity of test/NC diet was provided to the mice for *ad libitum* consumption at all times.

The mice were weighed and faecal pellets collected during the course of the experiment as outlined in Table 7.2. At these time points faecal pellets were obtained from each mouse and stored at -80 °C individually for 16S RNA sequencing. For MALDI TOF mass spectroscopy, HPLC and activity assays composite faecal samples were obtained by pooling the faecal pellets by cage at each time point. To limit contamination of the samples, the faecal pellets were collected directly from the mice and not from the bedding.

Table 7.2. Feeding schedule and days of faecal pellet collection and mouse and food hopper weighing.

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Faecal pellet collection	✓				✓				✓			✓			✓	
Mice weighed	✓	✓		✓				✓			✓					✓
Food hopper weighed	✓	✓		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Hours on test diet	2	2	2	4	4	4	4	4	6	6	6	8	8	8	8	n/a
Hours on nutritionally complete diet	22	22	22	20	20	20	20	20	18	18	18	16	16	16	16	n/a

7.3.5. DNA extraction, amplification and sequencing

DNA was extracted from faecal pellets using a QIAamp® Fast DNA Stool Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions with some modifications. To increase DNA yield, after the addition of InhibitEX buffer, bead beating (3 min × 2) and an incubation at 95 °C for 5 min, were performed. The samples were quantified using a Qubit® dsDNA High Sensitivity Assay Kit (Fisher Scientific, Dublin, Ireland) in conjunction with a Qubit® 2.0 fluorometer (Invitrogen,

Paisley, UK). The initial amplification PCRs were performed as outlined in the Illumina 16S Metagenomic Sequencing Library Preparation Guide (Illumina, Saffron Walden, UK) with the following alterations; 30 amplification cycles were used and the amplification PCRs were each performed in a total volume of 60 µL which contained 25 ng DNA and 1 µL of each primer at a 10 µM concentration. The subsequent clean up using the AMPure® XP purification system (Labplan, Dublin, Ireland) was scaled up appropriately to account for the greater volume. The index PCRs and subsequent AMPure® XP clean up were as outlined in the Illumina protocol. The samples were quantified using the Qubit® procedure and the concentrations normalized to 20 nM and pooled as per the Illumina protocol. The pooled sample (100 µL) was purified using AMPure® XP beads and the sample eluted using 50 µL of a 10 mM Tris solution. The pooled sample was quantified using the Qubit® procedure and sample quality was determined using an Agilent 2100 Bioanalyzer (Agilent, Cork, Ireland). The pooled sample was denatured and sequenced using a 500 cycle v2 kit on the MiSeq™ sequencing platform (Illumina, Saffron Walden, UK) following protocols outlined by Illumina, at the Teagasc Sequencing Centre, Moorepark.

7.3.6. Bioinformatics analysis

Sequences were filtered on the basis of quality (removal of low quality nucleotides at the 3' end) and length (removal of sequences with less than 200 nt) with PRINSEQ (Schmieder & Edwards, 2011) and joined using fastq-join (Aronesty, 2011). The sequences were clustered with 97% identity level (calculated at the

operational taxonomic unit; OTUs) using closed-reference USEARCH v7.0 algorithm (Edgar, 2010) against the Ribosomal Database Project (Wang, Garrity, Tiedje, & Cole, 2007). Alpha and beta-diversity was determined using QIIME (Caporaso et al., 2010). The results of principal coordinates analysis (PCoA) of the beta-diversity when it was calculated using distance matrices built from unweighted UniFrac distances, were visualized using EMPeror (Vazquez-Baeza, Pirrung, Gonzalez, & Knight, 2013).

7.3.7. Preparation of faecal pellets for detection of nisin

To detect nisin in the faecal pellets, the nisin was extracted from the pellets as described by Rea et al. (2014) with minor modifications as follows: composite faecal samples were suspended in 1 mL of 0.1% TFA and 70% IPA, vortexed thoroughly and allowed to stand at room temperature for 30 min and centrifuged for 5 min at 16,000 × *g* and the supernatant retained. The centrifugation step was repeated a further three times with the supernatant retained each time. In order to bring the IPA content of the samples to <7%, IPA was removed using a Centrivap Console (Labconco, Kansas City, US) and the samples were then restored to their original volumes using 0.1% TFA.

7.3.8. Reversed phase - high performance liquid chromatography (RP-HPLC)

RP-HPLC was carried out on a Jupiter, 5 μm, C18, 300 Å, 250 mm × 4.6 mm column from Phenomenex (Macclesfield, UK) with an acetonitrile (Thermo Fisher Scientific, Dublin, Ireland) gradient as described previously (Gough, Gómez-Sala, et al., 2017).

7.3.9. Matrix-assisted laser desorption/ionization time of flight mass spectroscopy (MALDI TOF MS)

The molecular mass of the HPLC fraction corresponding to the nisin peak was determined using MALDI TOF MS using an Axima TOF^2 (Shimadzu Biotech, Kyoto, Japan) as previously described (Field et al., 2012).

7.3.10. Activity assay

Antibacterial activity was estimated by agar diffusion activity assays (Ryan, Rea, Hill, & Ross, 1996) in agar plates seeded with *Lactococcus lactis* subsp. *cremoris* HP as described previously (Gough, Gómez-Sala, et al., 2017). Nisin was extracted from the faecal pellets as described above and Tween® 80 was added to a final concentration of 1% to prevent nonspecific adsorption of the nisin. The samples were dispensed into the wells of the seeded agar in 50 μL aliquots and the plates incubated overnight at 30 °C. Antibacterial activity resulted in zones of inhibition

surrounding the wells. Nisin was quantified based on a published method (Bernbom et al., 2006) by plotting the area of the zone of inhibition against the log of the nisin concentration of a serial dilution of Nisaplin® that was suspended in an equivalent solution to the samples (6% IPA, 0.1% TFA, 1% Tween® 80), to generate a linear standard curve.

7.3.11. Statistical analysis

Data was tested for normality of distribution using the Shapiro-Wilk test. For comparing two groups Student's *t*-test or Mann-Whitney *U* test were used as appropriate and for comparison of multiple groups one-way ANOVA or Kruskal-Wallis test were used as appropriate, additionally analysis of beta diversity was performed using the Adonis function in the R package Vegan (Oksanen et al., 2015). Analysis of the bioinformatics data was performed using the R statistical package (R Core Team, 2015) and all other analysis was performed using the SigmaStat software (Systat Software, San Jose, US). Results are expressed as mean ± standard error.

7.4. Results

7.4.1. Quantity of diets consumed and effect on weight gain

The cumulative consumption of the NC and test diets and resultant body weight gain are shown in Fig. 7.1. There were no significant differences in body

weight gain or in NC diet consumption between diet groups over the trial period with three exceptions, each of which occurred only at a single measurement time point; the consumption of the NC partner diet for SD and SG-N in the 6 h consumption period was significantly different (p = 0.02, n = 6), the weight gain for the mice on the SG and SG-N diets from days 4 to 7 of the trial was significantly different (p = 0.02, n = 10) and the weight gain for the mice on the SD and SG diets from days 11 to 15 of the trial was significantly different (p = 0.0004, n = 10). The total consumption per cage of the SD-N and SG-N test diets was 20.8 ± 2.5 g and 6.5 ± 2.0 g, respectively, and the daily consumption of these diets were significantly different during the 6 h (p = 0.00007, n = 6) and 8 h (p = 0.00003, n = 8) consumption period. The total nisin consumption per cage over the course of the trial was 144 ± 14 mg and 52 ± 11 mg for the SD-N and SG-N diet groups, respectively, and the daily consumption of the nisin portion of those diets were also significantly different during the 6 h (p = 0.0003, n = 6) and 8 h (p = 0.00002, n = 8) consumption period. The average nisin consumption per day per cage during the 8 h consumption period was 17 ± 1 mg and 6 ± 2 mg for the SD-N and SG-N diets groups, respectively. Therefore there was approximately a threefold greater consumption of nisin by mice on the SD-N diet compared to mice on the SG-N diet. For SD-N compared to SD, SG-N compared to SG and SD compared to SG there were no statistically significant differences during the 6 h (p = 0.134, 0.101 and 0.217 respectively, n = 6) and 8 h (p = 0.507, 0.442 and 0.54, n = 8) consumption periods (days 9 to 15 of the trial).

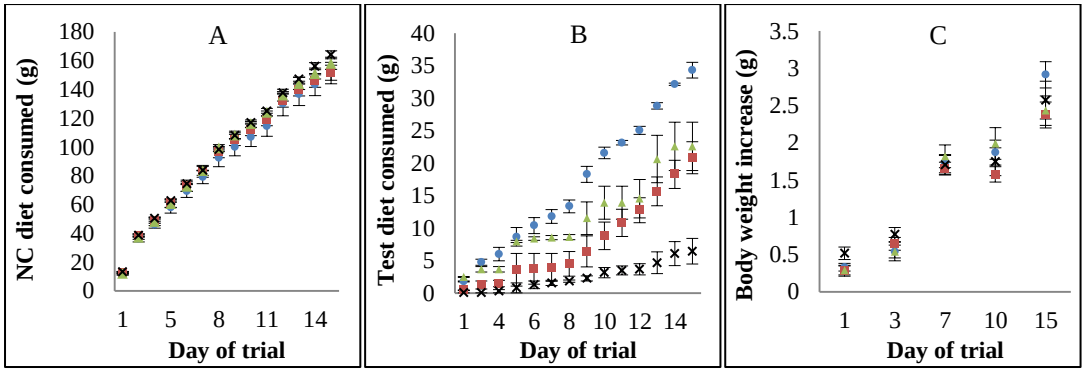

Fig. 7.1. Consumption of each diet and the relationship between diets and weight gain. (**A**) cumulative consumption of nutritionally complete (NC) partner diets for each diet group, (**B**) cumulative consumption of test diets for each diet group, (**C**) cumulative weight gain for each diet group. Diet groups are defined by their test diet as follows: ● starch dough (SD), ■ starch dough containing nisin (SD-N), ▲ starch gel (SG), ✕ starch gel containing nisin (SG-N).

7.4.2. Identification and quantification of intact nisin and nisin fragments in the faeces

The activity assays of the faecal pellets from mice consuming SD, SD-N, SG and SG-N diets (Fig. 7.2A) showed antibacterial activity in faeces from mice that consumed the SD-N and SG-N diets. MALDI TOF MS was performed on faecal pellets to determine their intact nisin and nisin fragment composition (Fig. 7.2B and C). Their primary nisin components were then determined by RP-HPLC in conjunction with MALDI TOF MS (Fig. 7.2D and E). For comparison purposes intact nisin was also analysed by RP-HPLC in conjunction with MALDI TOF MS (Fig. 7.2F and G).

MALDI TOF MS of the faecal pellets of mice on the SD-N (Fig. 7.2B) and SG-N diets (Fig. 7.2C) showed masses that correlated with intact nisin and nisin fragments 22-31 (i.e., corresponding to amino acids 22 to 31 of intact nisin) and 21-31; these nisin fragments are the products of the digestion of nisin and have predicted molecular masses of 1063.47 Da and 1195.44 Da, respectively (Slootweg,

Liskamp, & Rijkers, 2013). Versions of nisin fragment 22-31 with a Na adduct ion (+22 Da) and a K adduct ion (+38 Da) were also detected. Intact nisin, extracted from the faecal pellets, was seen in its doubly charged form at 1676.46 Da and 1675.61 Da for the SD-N diet and for the SG-N, respectively.

RP-HPLC of the faecal pellets of mice on the SG-N diet showed a single dominant peak (Fig. 7.2D) that eluted at 41% acetonitrile and MALDI TOF MS of this peak revealed it to be nisin fragment 22-31 (Fig. 7.2E). A similar result was obtained for the faecal pellets of mice on the SD-N diet (result not shown). Therefore, the primary nisin component of the faeces was fragment 22-31, as opposed to intact nisin.

Intact nisin normally elutes from a RP-HPLC at 36% acetonitrile (Fig. 7.2F) and subsequent MALDI TOF MS of this elution peak shows both singly (3354.46 Da) and doubly (1677.68 Da) charged intact nisin (Fig. 7.2G). However, while no intact nisin was detected by HPLC, antibacterial activity was detected in the faeces of those groups fed the SD-N and SG-N diets (Fig. 7.2A). This would suggest that the nisin concentration in the faecal pellets was below the level of detection by HPLC.

Quantifying the intact nisin in the faeces at the final time point based on antibacterial activity showed significantly more ($p = 0.031$, $n = 3$) nisin in the faeces of the group fed SG-N (1.7 ± 0.2 ng/mg) compared to the groups fed SD-N (0.8 ± 0.1 ng/mg), despite the fact that less nisin was consumed by the group fed the SG-N diet, which would indicate that more intact nisin reached the lower GIT in SG-N-fed mice. Therefore, at the final time point (8 hour test diet period), despite the significantly ($p = 0.00002$, $n = 8$) greater nisin consumption of the mice on the SD-N

diets, there was significantly (p = 0.031, n = 3) greater amount of nisin in the faeces from consumption of the SG-N diets.

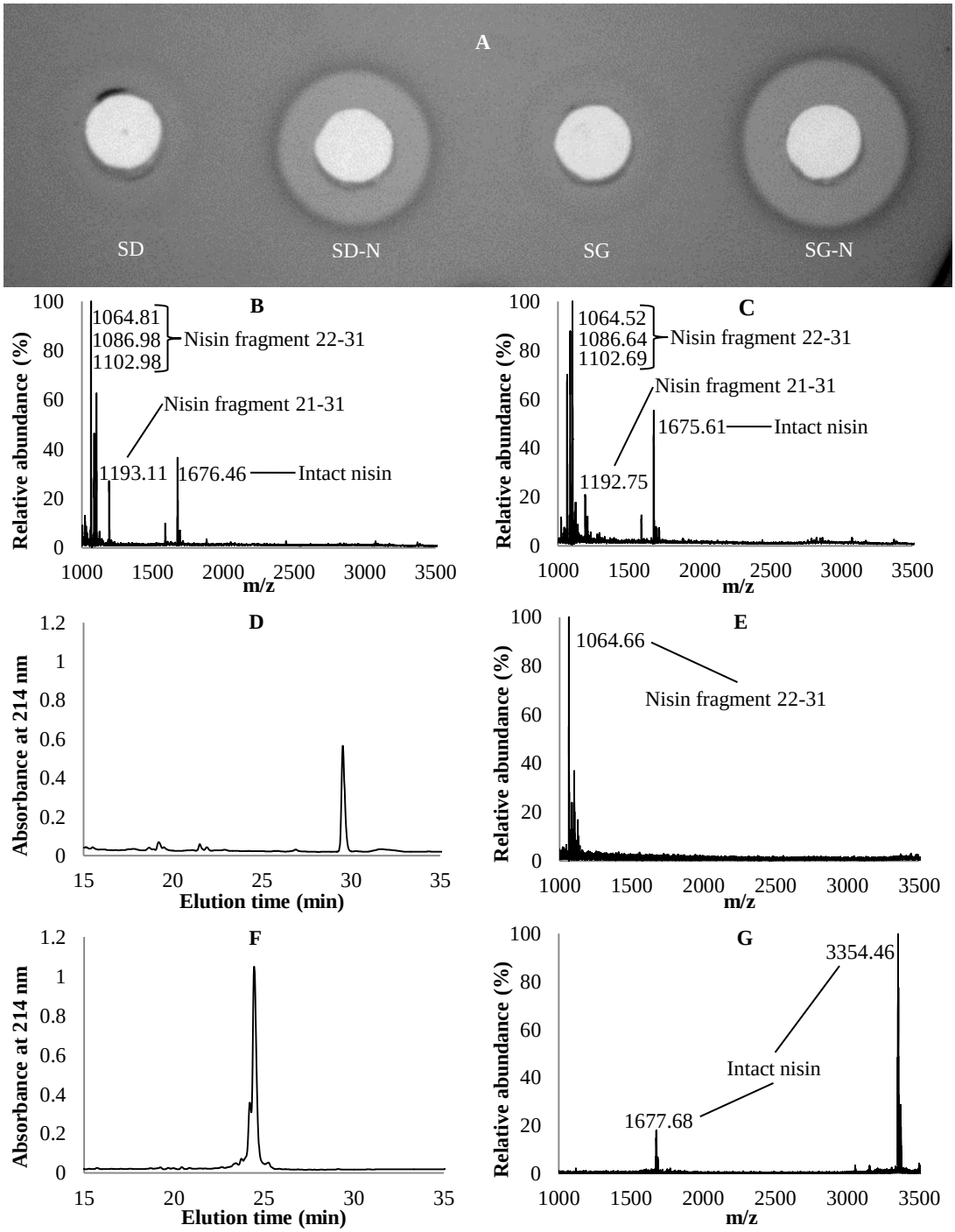

Fig 7.2. Analysis of faecal pellets of mice consuming starch dough (SD), starch dough containing nisin (SD-N), starch gel (SG) and starch gel containing nisin (SG-N) diets. Activity assay of faecal pellets from mice consuming SD, SD-N, SG and SG-N diets (**A**). Mass spectroscopy of faecal pellets from mice consuming SD-N (**B**) and SG-N diets (**C**). RP-HPLC chromatogram of faecal pellets from mice consuming SG-N (**D**) and mass spectroscopy of the elution peak (**E**). RP-HPLC chromatogram of intact nisin (**F**) and mass spectroscopy of the elution peak (**G**).

7.4.3. HTS-based analysis of microbiota

Following total metagenomic DNA extraction from the faecal pellets from day 15, 16S rRNA gene amplicons (V3-V4 region) were generated and sequenced using the Illumina MiSeq™ platform. The mean number of sequence reads and alpha diversity indices for each diet group are shown in Table 7.3. There were no statistical differences in the alpha diversity indices: Observed operational taxonomic units (unique operational taxonomic units), Chao1 (richness), ACE (richness), Simpson (richness and evenness) and Shannon (richness and evenness), between the diet groups (Table 7.3). However, when the beta diversity was calculated using distance matrices built from unweighted UniFrac distances and the PCoA results visualized using EMPeror (Vazquez-Baeza et al., 2013), the four treatment groups formed distinct clusters based on diet (Fig. 7.3), which were significantly different (p = 0.001, n = 10).

Table 7.3. Mean sequence reads and alpha diversity indices for starch dough (SD), starch dough containing nisin (SD-N), starch gel (SG) and starch gel containing nisin (SG-N) diet groups (mean ± standard error, n = 10).

	SD	SD-N	SG	SG-N
Sequence Reads	43,465 (± 7,276)	52,311 (± 4,629)	39,848 (± 3,909)	42,903 (± 4,969)
Observed Operational Taxonomic Units	267 (± 19)	300 (± 12)	246 (± 11)	296 (± 22)
Chao1	277 (± 18)	309 (± 12)	254 (± 12)	303 (± 22)
ACE	279 (± 18)	310 (± 12)	256 (± 11)	304 (± 22)
Shannon	3.58 (± 0.03)	3.58 (± 0.05)	3.57 (± 0.04)	3.69 (± 0.10)
Simpson	0.947 (± 0.002)	0.941 (± 0.005)	0.947 (± 0.003)	0.940 (± 0.007)
Inverse Simpson	19.1 (± 0.8)	17.8 (± 1.2)	19.4 (± 1.0)	18.7 (± 2.2)

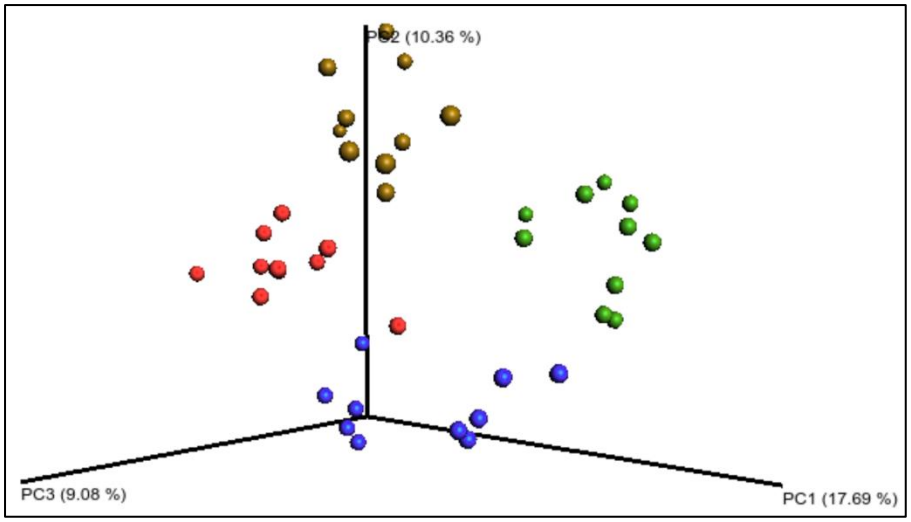

Fig. 7.3. Principal coordinates analysis (PCoA) of the unweighted UniFrac distances of the 16S sequencing data. The four diet groups are represented by colored circles: blue – starch dough (SD), green – starch dough containing nisin (SD-N), red – starch gel (SG), brown – starch gel containing nisin (SG-N). The groups are significantly different (p = 0.001, n = 10).

Sequence analysis revealed that the microbiota were primarily comprised of six phyla and that *Bacteroidetes* and *Firmicutes* were the dominant phyla showing a relative abundance of 54-62% and 25-33% respectively. There were no significant differences between the relative abundance of *Bacteroidetes* and *Firmicutes* across the diet groups (Fig. 7.4), however there were significant differences (p < 0.001, n = 10) in the relative abundance between diet groups in the phyla *Actinobacteria*, *Tenericutes* and *Verrucomicrobia* (Fig. 7.4).

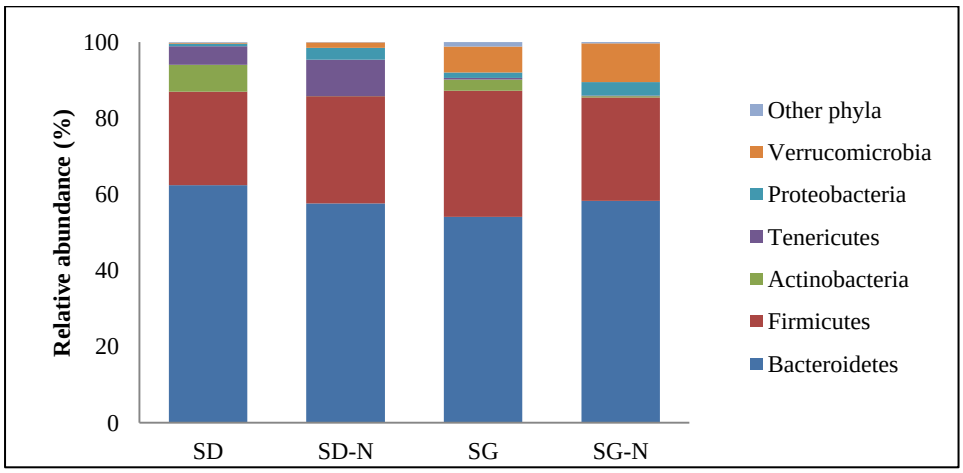

Fig. 7.4. Relative abundance at phylum level with respect to each diet. Diet groups are as follows: starch dough (SD), starch dough containing nisin (SD-N), starch gel (SG), and starch gel containing nisin (SG-N) diet (n = 10).

A number of statistical differences were found at genus level between the different dietary groups (Fig. 7.5 and Table 7.4). The mice fed the SG-N diet had significantly lower relative abundance of the genera *Allobaculum*, *Bifidobacterium*, *Lachnospiracea* incertae sedis and *Clostridium* cluster XIVa and significantly higher relative abundance of the genera *Escherichia/Shigella*, *Lactococcus* and *Corynebacterium* compared to the mice fed the SG diet ($p < 0.001$, $n = 10$). These changes were reflected at the corresponding family level. However, there was also a significantly higher ($p = 0.0005$, $n = 10$) relative abundance of the family *Ruminococcaceae* (Table 7.4) in mice fed the SG-N diet that did not correspond to a significant increase of any genus related to the *Ruminococcaceae* family. This likely reflects the combined increases (not individually statistically significant) in the proportions of the genera *Anaerotruncus* and *Hydrogenoanaerobacterium*, i.e., members of the *Ruminococcaceae* family, in mice that consumed the SG-N diet. Relative to the SD diet, the SD-N diet significantly ($p < 0.001$, $n = 10$) affected the relative abundance of only three genera; i.e. *Lactobacillus* and *Bifidobacterium*, which were lower, and *Escherichia/Shigella*, which were higher (Table 7.4).

There were also differences between the diet groups when compared on the basis of starch matrix. The relative abundance of the genera *Anaeroplasma*, *Bifidobacterium* and *Odoribacter* were significantly ($p < 0.001$, $n = 10$) greater in the mice fed the SD diet compared to the SG diet, whereas the relative abundance of the genera *Akkermansia*, *Lachnospiracea* incertae sedis and *Parabacteroides* were all significantly ($p < 0.001$, $n = 10$) greater in the mice fed the SG diet relative to the SD diet (Fig. 7.5 and Table 7.4). In addition, *Clostridium* cluster XIVb and *Desulfovibrio* had greater relative abundance in the mice fed the SG and SG-N diets

compared to the mice fed the SD and SD-N diets (Table 7.4) and this was significantly different ($p < 0.001$, $n = 10$) for the SG-N diet group compared to the SD-N diet group.

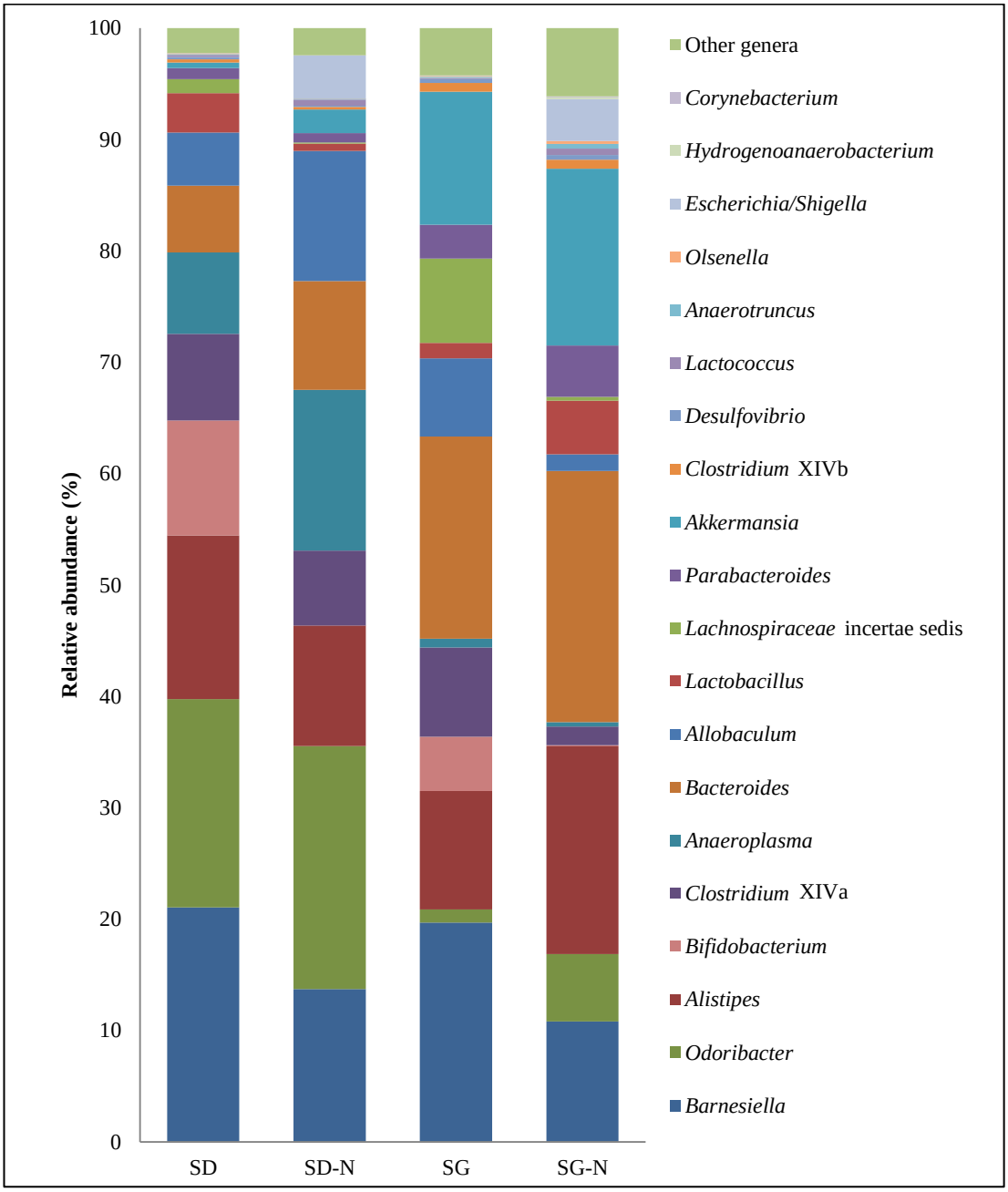

Fig. 7.5. Relative abundance at genus level with respect to each diet. Diet groups are as follows: starch dough (SD), starch dough containing nisin (SD-N), starch gel (SG), and starch gel containing nisin (SG-N) diet ($n = 10$).

Table 7.4. Bacterial taxa whose relative abundance was significantly different between diet groups. Diet groups are as follows: starch dough (SD), starch dough containing nisin (SD-N), starch gel (SG) and starch gel containing nisin (SG-N). The relative abundance of each bacterial taxon is expressed as mean ± standard error. The same letter after a pair of values in a single row indicates these values are significantly different ($p < 0.001$, $n = 10$): (a) SD compared to SG, (b) SD-N compared to SG-N, (c) SD compared to SD-N and (d) SG compared to SG-N.

	SD	SD-N	SG	SG-N
Significant at genus level				
Akkermansia	0.499 (± 0.111) a	2.120 (± 1.077)	11.943 (± 2.369) a	15.879 (± 4.789)
Allobaculum	4.764 (± 0.827)	11.690 (± 2.107) b	7.018 (± 1.245) d	1.504 (± 0.528) bd
Anaeroplasma	7.307 (± 1.905) a	14.442 (± 2.667) b	0.799 (± 0.180) a	0.385 (± 0.174) b
Bifidobacterium	10.317 (± 0.902) ac	0.005 (± 0.001) bc	4.894 (± 0.602) ad	0.090 (± 0.030) bd
Clostridium cluster XIVa	7.777 (± 1.044)	6.731 (± 0.577) b	8.003 (± 1.261) d	1.685 (± 0.646) bd
Clostridium cluster XIVb	0.289 (± 0.095)	0.226 (± 0.045) b	0.777 (± 0.117)	0.831 (± 0.145) b
Corynebacterium	0.007 (± 0.002)	0.021 (± 0.007)	0.007 (± 0.003) d	0.091 (± 0.018) d
Desulfovibrio	0.222 (± 0.058)	0.119 (± 0.031) b	0.350 (± 0.076)	0.415 (± 0.058) b
Escherichia/Shigella	0.009 (± 0.005) c	3.860 (± 1.971) c	0.011 (± 0.002) d	3.771 (± 1.795) d
Lachnospiraceae incertae sedis	1.248 (± 0.375) a	0.126 (± 0.091)	7.579 (± 1.410) ad	0.336 (± 0.281) d
Lactobacillus	3.536 (± 0.470) c	0.643 (± 0.330) c	1.378 (± 0.351)	4.828 (± 3.140)
Lactococcus	0.168 (± 0.024)	0.533 (± 0.080)	0.095 (± 0.014) d	0.580 (± 0.143) d
Odoribacter	18.711 (± 2.515) a	21.817 (± 1.322) b	1.164 (± 0.186) a	6.058 (± 2.205) b
Parabacteroides	0.996 (± 0.160) a	0.835 (± 0.108)	3.042 (± 0.662) a	4.597 (± 1.200)
Not significant at genus level but significant at family level				
Ruminococcaceae	2.285 (± 0.468)	3.296 (± 0.420)	2.157 (± 0.273) d	7.759 (± 0.684) d
Unclassified *Ruminococcaceae*	1.519 (± 0.356)	2.228 (± 0.259)	1.492 (± 0.201) d	5.284 (± 0.423) d

7.5. Discussion

The aim of this study was to determine if orally ingested nisin could be delivered to the lower GIT in two different starch matrices and subsequently impact on the lower GIT microbiota. Additionally, it was examined whether the type of starch itself could modulate the lower GIT microbiota.

To the authors' knowledge (Table 7.1) the only study that has examined the effect of orally ingested nisin on the rodent microbiota is the study on rats by Bernbom et al. (2006), in which the highest amount of nisin consumed was 174 mg nisin per kg body weight per day and, while nisin was detected in the faeces, no

changes in the microbiota were detected which may be due to the sensitivity of the molecular methods used in that study. In this study it was hoped that using a 16s HTS approach and similar levels of nisin as described by Bernbom et al. (2006), it would be possible to determine the impact of nisin on the microbiota. All test diets were increased at intervals over the trial to acclimatize the mice to consuming starch and nisin. At the 8 h consumption period the mice consumed 161 and 54 mg nisin per kg body weight per day for the SD-N and SG-N diets respectively. To limit the stress on the mice they were allowed unrestricted access to a diet within a given consumption period, however this approach limited a more precise matching of the amount fed to the Bernbom et al. (2006) study.

Numerous studies have shown that nisin is susceptible to digestion by the enzymes in the upper GIT and a previous study by our group using the *in vitro* INFOGEST digestion model for the human GIT, detected nisin fragments corresponding to the N-terminus of nisin (amino acids 1-11, 1-12, 1-20, 1-21, 1-29 and 1-32) post digestion, while no intact nisin was detected (Gough, O'Connor, et al., 2017). In this study, low levels of biologically active nisin (ng/mg of faeces) were detected in the faeces of mice fed SG-N and SD-N, but, in contrast to the *in vitro* study, the primary nisin component of the faeces was fragment 22-31, which is not biologically active as the N-terminus is required for nisin activity (Hsu et al., 2004). It is also notable that the fragments produced by the *in vivo* digestion had a significant portion of their C-terminal present whereas those produced by the *in vitro* digestion had a significant portion of their N-terminal present. These differences can most likely be attributed to species-related differences in digestive enzymes.

More nisin was detected in the faecal samples of the mice on the SG-N diet despite them having consumed less nisin than those on the SD-N diet; implying that

more intact nisin reached the lower GIT on the SG-N diet and that the starch gel may have afforded some protection to the nisin from digestion in the upper GIT. To the authors' knowledge there are no reported studies of the *in vivo* effect of nisin on the gut microbiota using HTS techniques. The results of 16S rRNA compositional sequencing showed that alpha diversity indices for all diet groups were comparable to those seen in previous studies on faecal samples from C57BL/6J mice on low-fat diets (Allen et al., 2015; Javurek et al., 2017). Notably, however, beta diversity analysis showed that the murine microbiotas clustered together on the basis of diet. With respect to taxonomy, significant differences in the relative abundance between diet groups were observed for the phyla *Actinobacteria*, *Tenericutes* and *Verrucomicrobia* ($p < 0.001$, $n = 10$). In each case a single genus, i.e. *Bifidobacterium*, *Anaeroplasma* and *Akkermansia*, respectively, comprised the majority (>98%) of the genera detected belonging to these phyla.

There were differences between the cumulative consumption of SD compared to SG (Fig. 7.1B), however as detailed above, those differences were not statistically significant for the 6 h and 8 h consumption periods (days 9 to 15 of the trial). Resistant starch is known to effect satiety (Lockyer & Nugent, 2017). The hormones glucagon-like peptide 1 (GLP-1) and peptide tyrosine-tyrosine (PYY) which are involved in the regulation of satiety and glycemic response (D'Alessio, 2008) have been demonstrated to be elevated by resistant starch consumption in studies on mice (Zhou et al., 2008). Although to the authors knowledge while the relative ability of difference resistant starch types to effect satiety has not been elucidated, it has been shown that different types of resistant starch elicit significantly different glycemic responses (Haub et al., 2010). Therefore it may be possible that the differences in consumption of the SD and SG are due to differences

in the effect of RS2 and RS3 on satiety, however this was not investigated further in this study given our focus on the effects of nisin on the gut microbiota.

There were also differences between the cumulative consumption of SG-N compared to SG, and SD-N compared to SD (Fig. 7.1B), however as detailed above, those differences were not statistically significant for the 6 h and 8 h consumption periods (days 9 to 15 of the trial). This reduction in consumption is unlikely to be due to an effect of nisin on the microbiota as such a change in the microbiota would also affect the consumption of the NC diets and there were no statistically significant differences between the consumption of their respective partner NC diets throughout the trial (Fig. 7.1A).While high protein diets have been shown to increase satiety, the level of protein in the nisin containing diets (~0.58%) is unlikely to have had an effect on satiety in this case (Batterham et al., 2006; Wiessing, Xin, Budgett, & Poppitt, 2015; Yu, South, & Huang, 2009). However, it is possible that the palatability of nisin may have contributed to the reduced consumption of the nisin containing diets.

A limitation of this study is that the mice consumed different quantities of each of the test diets (Fig. 7.1B). While this could have confounded the effect of nisin on the microbiota of the diet groups when SD is compared to SD-N and SG is compared to SG-N (Table 7.4), the changes in the microbiota, nonetheless, are consistent with the specific effect of nisin on these microorganisms. The difference in the amount of starch consumed and resistant starch type could have confounded the effects of the nisin. However, when comparing diets containing dough and gel (Table 7.4), of the eight genera that showed a significant difference ($p < 0.001$, $n = 10$) in relative abundance, only two genera were also significantly different ($p < 0.001$, $n = 10$) in relative abundance when SD-N was compared to SD and SG-N was

compared to SG. Additionally, of these two genera, *Lachnospiraceae* incertae sedis was only significantly different ($p < 0.001$, $n = 10$) in relative abundance in SG-N compared to SG, while *Bifidobacterium* showed significantly lower ($p < 0.001$, $n = 10$) relative abundance in both the SD-N compared to SD and SG-N compared to SG. It has been reported previously that *Bifidobacterium* are particularly sensitive to nisin relative to other intestinal bacteria (Le Blay, Lacroix, Zihler, & Fliss, 2007). Furthermore nisin primarily targets Gram positive bacteria. Interestingly the genera that were significantly lower ($p < 0.001$, $n = 10$) in relative abundance in SD-N compared to SD and SG-N compared to SG (Table 7.4) were Gram positive or primarily Gram positive (*Clostridium* cluster XIVa), whereas the genera and family that significantly increased ($p < 0.001$, $n = 10$) in relative abundance in SD-N compared to SD and SG-N compared to SG (Table 7.4) were either Gram negative (*Escherichia/Shigella*), contained Gram negative members (*Ruminococcaceae*) or may have had nisin resistant mechanisms that are known to be present in some strains (*Lactococcus* and *Corynebacterium*) (Brenner, Krieg, Staley, & Garrity, 2005; De Vos et al., 2009; Draper, Cotter, Hill, & Ross, 2015; Gharsallaoui, Oulahal, Joly, & Degraeve, 2016; Goodfellow et al., 2012; Zhou, Fang, Tian, & Lu, 2014). Taking these points together, we hypothesize that one reason for the differences in relative abundance between SD-N compared to SD and SG-N compared to SG is the presence or absence of nisin in the test diets.

Starch based doughs have been proposed for use for the oral delivery of drugs to laboratory rodents as a stress free alternative to oral gavage (Corbett et al., 2012). We observed in preliminary *in vitro* studies, that SD-N when placed in water rapidly dissociated releasing the nisin, whereas SG-N did not dissociate and nisin release was limited. Therefore it is possible that the nisin would be released earlier

and more rapidly from the SD-N than from the SG-N, which would in turn result in more of the nisin being digested in the upper GIT by the digestive enzymes secreted there and therefore impacting less on the microbiota in the lower GIT than nisin incorporated into the SG-N. While it is acknowledged that there are difficulties discerning the effect of the rate of release of the starch matrices from the effect of the level of consumption and resistant starch type, the compositional sequencing provides some evidence that nisin was released from the SD-N early in GIT transit and from the SG-N late in GIT transit. The relative abundance of *Lactobacillus*, which are primarily residents in the upper GIT that in turn transiently populate the lower GIT (Denev, 2006; Walter, 2008), was reduced in the SD-N fed group but were unaffected in the SG-N fed group, which may point to an earlier release in the upper GIT resulting in fewer lactobacilli reaching the colon. Additionally the SG-N diet affected the relative proportion of more than three times as many genera that are primarily resident in the lower GIT than were affected by the SD-N diet (when comparing both with their respective 'starch only' controls); this indicates that the SG-N delivered more nisin to the lower GIT than the SD-N. Furthermore, despite there being approximately three fold lower consumption of nisin by the mice on the SG-N diet compared to the mice on the SD-N diet, there was approximately twice as much nisin detected in the faeces of the mice that consumed the SG-N diet compared to those that consumed the SD-N diet.

Bifidobacterium and *Escherichia/Shigella* were the only two genera significantly ($p < 0.001$, $n = 10$) different in both the SD-N and SG-N diet groups compared to the SD and SG diet groups. Bifidobacteria have been demonstrated to attenuate *Escherichia/Shigella* in several studies, including in mice (Cheikhyoussef, Pogori, & Zhang, 2007; Gibson & Wang, 1994; Shu & Gill, 2001). It is possible that

a nisin mediated reduction in the relative abundance of bifidobacteria allowed *Escherichia/Shigella* to increase in relative abundance; particularly as these were the only two genera resident in the lower GIT that were significantly different when the SD-N and SD diet groups were compared.

While it would be interesting to determine whether the changes in the microbiota seen in this study could occur at substantially lower levels of nisin consumption such as those found in food, a dose response study would be required to evaluate this. The current acceptable daily intake (ADI) is 1 mg nisin per kg body weight per day (Younes et al., 2017) while typical levels added to foods range from 2.5-25 mg/kg (Delves-Broughton, 2005).

Resistant starch is capable of modulating the microbiota in the lower GIT and its effect depends on the type of resistant starch (Bird, Brown, & Topping, 2000; Martinez et al., 2010). Many of the genera whose relative abundance was significantly different ($p < 0.001$, $n = 10$) when compared on the basis of resistant starch type including *Akkermansia*, *Anaeroplasma*, *Bifidobacterium*, *Lachnospiracea*, *Odoribacter* and *Parabacteroides* have positive health associations (Gomez-Gallego et al., 2016; Kverka et al., 2011; Leahy, Higgins, Fitzgerald, & van Sinderen, 2005; Noor et al., 2010; Reeves, Koenigsknecht, Bergin, & Young, 2012; Vital, Howe, & Tiedje, 2014; Zeng et al., 2015). Of particular interest was the alteration in the relative abundance of *Akkermansia* which has been described as a "next generation probiotic" (Cani & Van Hul, 2015) and is associated with numerous health benefits including treating type 2 diabetes, reducing the occurrence of autoimmune diseases and in weight management (Gomez-Gallego et al., 2016). *Akkermansia* in the murine gut is generally low (Schubert, Sinani, & Schloss, 2015). Diets that include resistant starch have been shown previously to increase the relative

abundance of *Akkermansia* (Tachon et al., 2013). There was less SG (RS3) consumed than SD (RS2) over the course of the study (Fig. 7.1B), although as detailed above, this difference in consumption was not significant during the 6 h or 8 h consumption periods (days 9 to 15 of the trial), however the relative abundance of *Akkermansia* was significantly ($p = 0.0002$, $n = 10$) greater in mice fed the SG (RS3) than the SD (RS2) diet (12 and 0.5% relative abundance respectively). This may be attributable to the type of starch, however confirmation of this would require further investigation using NC diets incorporating the various starch types.

Overall, while it may be possible to attribute the differences in the microbiota between the diet groups to the effects of the diet components, it is important to highlight that these may not all be direct effects. The GIT microbiota is an interdependent community and the effect of a diet component on members of that network may promote other members that were not directly affected by the diet component (Scott, Antoine, Midtvedt, & van Hemert, 2015; Willing, Russell, & Finlay, 2011).

Increased body weight gain due to nisin consumption has been demonstrated in previous studies involving chickens and rabbits (Table 7.1). However, in this study, no effect of nisin on body weight was observed, regardless of the matrix used for delivery. This is consistent with studies involving rats and quails and the majority of studies involving mice (Table 7.1).

7.6. Conclusions

This study showed that by using a starch matrix, nisin can be delivered to the lower GIT and will impact on the lower GIT microbiota. All four diets altered the

mouse microbiota differently, with the differences between the two nisin containing diets may be attributable to differences in how nisin was released and protected by the two starch matrices, while the differences between the starch matrices may be attributable to the type of resistant starch (type 2 and type 3) favouring the abundance of different bacterial taxa. It was particularly notable how the relative abundance of the probiotic *Akkermansia* differed between the two resistant starch diets however the difference in consumption between starch diets makes comparisons more difficult and this would need to be addressed in a further study. Despite greater consumption of the SD-N diet, the SG-N diet resulted in larger amounts of intact nisin in the faeces and appeared to affect a greater number of lower GIT bacterial taxa. This highlights the importance of the matrix when studying the activity of a bioactive peptide either as a food additive or as a therapeutic for gastrointestinal pathogens. This study also demonstrated, in an *in vivo* model, the usefulness of resistant starch, particularly in a retrograded gel, for the colonic delivery of a bioactive peptide. This system may be of use for other heat stable peptides, including those with a narrower range of antimicrobial activity.

7.7. References

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Chapter 8

General discussion

The aim of this study was to develop a system to allow oral delivery of a bioactive peptide to the colon. As a model peptide for testing the system, the antimicrobial peptide nisin was used as it is well characterised, commercially available and digested during gastrointestinal transit if taken orally. To achieve colonic delivery a starch matrix was used that resisted digestion in the upper gastrointestinal tract but could be digested by colonic bacteria.

Bioactive peptides have demonstrable health benefits treating a range of conditions including osteoporosis (calcitonin) (Karsdal et al., 2011), ulcerative colitis (ciclosporin) (Laharie et al., 2012) and *Clostridium difficile* infections (thuricin CD) (Rea et al., 2014). However when taken orally the majority of bioactive peptides, including the aforementioned peptides, have poor bioavailability (Karsdal et al., 2011; Keohane, Rosa, Coulter, & Griffin, 2016; Rea et al., 2014; Segura-Campos, Chel-Guerrero, Betancur-Ancona, & Hernandez-Escalante, 2011).

Despite there being a large range of food derived bioactive peptides (Hartmann & Meisel, 2007), there has been dearth of systems to ensure these functional food components are bioavailable (Mohan et al., 2015). Systems developed by the pharmaceutical industry tend to be complex and non clean-label, such as the systems developed for delivery of insulin by Diabetology and by Oramed Pharmaceuticals, which are comprised of a protective outer coat with pH triggered release, protease inhibitors and absorbance enhancers (Diabetology, 2017; Oramed Pharmaceuticals, 2018).

The colon is a privileged place for bioactive peptides due to the reduced protease activity, with a 20 to 60 fold decrease in protease activity relative to the small intestine (Washington, Washington, & Wilson, 2001). Additionally colonic delivery is essential for bioactive peptides that have a local effect in the colon,

particularly antibacterial peptides that can be used to modulate the colonic microbiota.

To the authors knowledge there is only one delivery system in commercial development for colonic delivery (ciclosporin, SmPill®, Keohane et al. (2016)) and there are currently no encapsulation based delivery systems being used commercially in food for bioactive food peptides (Mohan et al., 2015). It is possible that if a colonic based delivery system for bioactive peptides was sufficiently simple it may become commercially viable for use in the food industry.

The initial challenge in using a bioactive peptide is having a sufficient quantity of the peptide for study and in Chapter 2 a simple purification process was developed, based on a salting-out approach which allowed production of sufficient feedstock. This process produced a powder containing ~33% nisin, from a nisin producing culture and also enriched a commercial nisin preparation over 30-fold to a purity of ~58%. These are higher purities than comparable published methods, whereas the simplicity of the approach facilitates its use and scale-up.

To accurately gauge the protection offered by the delivery system it was necessary to model the digestion of nisin. *In vitro* digestion (Chapter 3) established which nisin fragments, several of which maintain a low level of antimicrobial activity, are produced by digestion. Although it has been previously established that there are nisin fragments with antibacterial activity (Chan et al., 1996) this is the first study to establish which nisin fragments occur under natural digestion conditions. It is noted that the nisin concentration used in this digestion was limited by the detection limits of the reversed phase - high performance liquid chromatography (RP-HPLC) approach used to analyse the nisin fragments; using a concentration closer to that found in food may have yielded different fragments. The European

Food Safety Authority (EFSA) have set the acceptable daily intake (ADI) of nisin to 1 mg/kg body weight (Younes et al., 2017) based primarily on toxicity studies in rats (Hagiwara et al., 2010). However, this toxicity study did not look at changes in the microbiota (Hagiwara et al., 2010). It is possible that these bioactive nisin fragments could negatively affect the microbiota without having a significant toxicological effect. Therefore in circumstances where these bioactive fragments of nisin would be present after a small intestinal digestion and reach the colon, the ADI may have to be re-evaluated to take into consideration their effect on the microbiota. In their most recent review on nisin (Younes et al., 2017) the EFSA acknowledged the findings of Chapter 3, that several of the products of nisin digestion have limited bioactivity, without further comment.

The presence or absence of bile in a digestion alters the relative proportions of the nisin fragments in the digestion products (Chapter 3). The presence of bile increases the portion of fractions with antibacterial activity. This highlights the importance, when modelling the digestion of a peptide, to include all the digestion components as opposed to just the proteases. We hypothesise that the reduced digestion of the N-terminal of nisin in the presence of bile is due to the insertion of the hydrophobic N terminal of nisin into the hydrophobic core of the bile micelle and thus gaining a degree of protection from proteases.

When starch (or its component polymers) are used as a protective coating for colonic delivery, they are normally combined with a binder, most commonly ethyl cellulose, to control swelling in aqueous solution and increase structural integrity and they are frequently applied by compression coating, spray coating and co-spray drying (Desai, 2007; Desai, 2005; Dimantov, Greenberg, Kesselman, & Shimoni, 2004; Freire et al., 2010; Krogars et al., 2003; McConnell et al., 2007; Milojevic et

al., 1996; Moussa & Cartilier, 1997; Palviainen et al., 2001; Pu et al., 2011; Recife, Meneguin, Cury, & Evangelista, 2017; Situ, Chen, Wang, & Li, 2014; Wilson & Basit, 2005). In Chapter 5 a simple starch gel approach was demonstrated to be capable of colonic delivery. In this approach, the peptides were blended with starch and water which was heated and cooled to form a starch gel. There was no additional binder, only the natural gelling capacity of the starch. The simplicity of this approach enhances its commercial applicability.

One straightforward way to improve on the efficiency of the gels produced in Chapter 5 would be to reduce the concentration of peptide. In this study the lowest amount of nisin used in the gels was 0.58% (w/w) due to the detection limitations of the reversed phase - high performance liquid chromatography (RP-HPLC) technique used to validate the gels. A lower proportion of peptide would likely result in a greater entrapment efficiency.

One limitation of the gel entrapment system is that it is only suitable for peptides that are heat tolerant. One approach would be to produce the gel using a lower temperature, however this would also likely result in a gel with poorer entrapment and digestion resistance due to less amylose being released from the granules. There are still many bioactive peptides that are known to be heat tolerant including antibacterials, antioxidants and antihypertensives (Casteels et al., 1989; Deraz et al., 2005; Singh & Vij, 2018).

To increase the range of peptides for which the starch gel delivery system could be used would be to include absorbance enhancers. This would allow the delivery of peptides that require systemic delivery. Many absorbance enhancers, such as the cell penetrating peptide, trans-activator of transcription (TAT) and the surfactant, tetradecyl maltoside (TDM), function better in the colon (Chen et al.,

2017; Petersen et al., 2013). However the inclusion of absorbance enhancers would take away from the clean-label low-cost ingredients and simplicity of the system. These are major strengths of the system as it is comprised of only starch and water and produced by simply heating and cooling.

In Chapter 6 nisin was labelled with Alexa Fluor® 647 Hydrazide to determine its localisation in the starch gel. The high stability of this particular label allowed it to remain bound to nisin and retain its fluorescence after the high heat and low pH of the gel entrapment process. The labelled nisin showed no antibacterial activity despite previous reports (Scherer et al., 2013) of this label binding nisin without affecting antibacterial activity. Additionally, the labelled nisin behaved differently to native nisin in ion exchange and hydrophobic interaction chromatography columns. Due to these differences between the native and labelled nisin, the localisation of the labelled nisin may not be representative of native nisin. There are other dyes in the Alexa Fluor® range that have a lower molecular mass and are less negatively charged than Alexa Fluor® 647; these may be more suitable for labelling nisin (Anderson & Nerurkar, 2002; Panchuk-Voloshina et al., 1999; Sobek, Aquino, & Schlapbach, 2011). Another approach would be to entrap native nisin in a starch gel, section the gel and then label the nisin, such as in the approach by Laridi et al. (2003). The surface labelling of the sections may limit the information on the three dimensional localisation of the nisin, however as the nisin is entrapped in a native state, this approach could be more accurate in determining the localisation of nisin in the starch gel.

In Chapter 7 it was seen in a mouse model that the entrapment of nisin in a starch gel enhanced its colonic delivery relative to a control comprising of nisin in a starch dough. Despite threefold greater nisin consumption on the control diet, the

nisin in starch gel diet resulted in the relative abundance of three times as many genera from the lower gastrointestinal tract (GIT) being significantly different ($p < 0.001$, $n = 10$) and twice as much nisin was detected in the faeces compared to the control diet. In Chapter 7 the mice consumed less nisin per kg body weight on both nisin in starch diets than the aforementioned rat trial (Hagiwara et al., 2010) that the EFSA used to set the current ADI (Younes et al., 2017). However, in both nisin in starch diets (Chapter 7), the microbiota was affected by nisin, whereas the effect on the microbiota was not examined in the Hagiwara et al. (2010) study. Further studies would be required to establish the greatest amount of nisin that can be consumed without affecting the microbiota.

It is well documented that the resistance starch content of a diet effects the composition of the colonic microbiota (Maier et al., 2017). There are 4 types of resistant starch based on whether their resistance is due to physical inaccessibility, granular structure, retrogradation or chemical modification (types 1, 2, 3 and 4 resistant starch respectively) (Sajilata, Singhal, & Kulkarni, 2006). There has been limited study of the difference in effect on the microbiota between different starch types (Martinez et al., 2010), or between polymorphs of a type, such as different thermal processing resulting in different type 3 structures (Lesmes et al., 2008). In Chapter 7 the type of resistant starch effected the microbial composition of the lower gastrointestinal tract of mice. Of particular interest was the probiotic *Akkermansia* which is known to increase in diets that include resistant starch (Gomez-Gallego et al., 2016; Tachon et al., 2013). In Chapter 7 the relative abundance of *Akkermansia* was 0.5% and 11.9% on the diets containing type 2 and 3 resistant starch respectively which is significantly different ($p = 0.0002$, $n = 10$).

To accurately establish the health benefits of resistant starch the relationship between an effect and the type and polymorph of the resistant starch needs to be established. The 16S rRNA compositional sequencing approach used in this study would be an ideal system to study how resistant starch types and polymorphs affect the microbiota of the lower gastrointestinal tract. Additionally, as the starch could be included as a component in the standard (nutritionally complete) diet, the diets would be simpler to setup and compare than in Chapter 7.

It is hoped that the system developed during the course of this thesis will enable further study into the use of food grade matrices to enhance the health benefits of bioactive peptides.

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