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Biomedical applications for bacteriocins in infection and oncology

A Thesis Presented to the National University of Ireland

for the Degree of

Doctor of Philosophy

by

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December 2018

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I hereby declare that this thesis is my own work and has not been submitted for another degree, either at University College Cork or elsewhere.

Signed _____

Jenna-Claire Ellis

Abstract

Lantibiotics are a subclass of a group of bacterially produced antimicrobial peptides called bacteriocins. They are characterized by post-translation modifications resulting in the presence of unusual amino acid residues such as dehydroalanine (Dha), dehydrobutyrine (Dhb) and lanthionine/methyllanthionine. Interest in using bacteriocins as alternative therapeutics in multiple settings has grown in recent years. Nisin in particular has been the subject of many studies due to it being well characterised, approved by the FDA, widely used as a food preservative (EU number E234) and having GRAS (generally regarded as safe) status. Nisin has bioengineered to generate multiple variants with advantageous properties, including enhanced antimicrobial activity. Nisin is also capable of inducing its own production (auto-induction) through the LisRK sensor kinase system. In Chapter 2 we examine the self-induction properties of a previously created nisin variant "nisin-AAA" by substituting positions K12 and H31 independently, revealing that ten variants retained the ability to induce the P_{nisA} promotor (K12K/H31H, K12V, K12Q, K12W, K12T, K12A, K12C, H31N, H31K and H31R). Amino acid substitutions at positions K12 and H31 were also made simultaneously generating the variant K12V-AAA-H31V, a variant previously shown to be trypsin and chymotrypsin resistant, but which lost its capacity for self-induction. Our results confirm that amino acid substitutions R, N and K at H31 are the only substitutions capable of maintaining auto-induction. Given that all three are cut sites for trypsin or chymotrypsin we conclude that any substitution at position H31 of the nisin-AAA variant will lead to either loss of auto-induction or a peptide which is sensitive to digestion by chymotrypsin.

The number of clinically significant bacteria becoming antibiotic resistant is increasing, along with concerns of pandrug-resistance (resistance to all current drugs),

emphasizing the need to introduce new therapeutics into the clinic. In Chapter 3, following an initial screening involving nisin, lacticin 3147 and several other bacteriocins, it was observed that bacteriocins could enhance the antimicrobial activity of classical antibiotics against selected clinically significant bacteria. Levels of antibiotics often associated with adverse effects and antibiotic resistance could be reduced in the presence of either lacticin 317 of nisin Z. Enhanced antimicrobial activity was seen between lacticin 3147 and penicillin G as well as vancomycin. In addition, methicillin (which is no longer used very much in clinical settings) showed partial synergistic activity in combination with nisin Z, suggesting the possibility of reviving old therapeutics with the aid of new antimicrobials.

Given that the anti-cancer effects of bacteriocins have been largely unexplored, in Chapter 4 we investigate the potential use of nisn Z as an alternative treatment for colorectal and oesophageal cancers, which have with high incidence and poor prognosis. It was observed by morphological examination that nisin Z elicited apoptotic cell death in 4 colorectal cancer cell lines and potentially autophagy mediated cell death in 2 oesophageal cancer cell lines. Confocal microscopy analysis along with flow cytometry profiles further supported autophagic cell death in oesophageal cell lines, whereas apoptotic cell death was supported in colorectal cell lines by flow cytometry. Upon further analysis it was observed that one colorectal cancer cell line, HCT116, initiated apoptotic cell death through the intrinsic pathway, as inferred by the upregulation of the protein caspase-9.

As a consequence of the studies presented in this thesis, it is possible that strategies will emerge to facilitate the use of bacteriocins such as nisin Z and lacticin 3147 in clinical settings for the treatment of multiple conditions.

Chapter I

Biomedical applications for bacteriocins in infection and oncology: review

Jenna-Claire Ellis, R. Paul Ross, Colin Hill

1.1 Summary

Dramatic increases in the incidence of clinically significant antibiotic resistant bacteria and the occurrence of gastrointestinal cancers demands that novel solutions should be explored. One avenue that has gained growing interest is the use of certain bacterially produced antimicrobial peptides called bacteriocins, such as nisin and lacticin 3147. Nisin and lacticin 3147 are both lantibiotics, a class of bacteriocins that are post-translational modified and contain unusual lanthionine/methyllanthionine residues, among others. Given that few cases of naturally occurring lantibiotic resistance have been recorded, taken together with their antimicrobial activity at low concentrations, indicates their possible use as an alternative therapeutic to conventional antibiotics should be further examined. In addition, more recent studies have demonstrated anti-cancer effects with certain bacteriocins selectively binding to cancer cells, suggesting they may have a place in future oncological therapeutic strategies. Nisin has demonstrated considerable anti-cancer activity *in vitro* and *in vivo*, suggesting a possible use in human therapy. Nisin has also been granted GRAS (generally regarded as safe) status which, together with its status as one of the most characterised and well-studied lantibiotics to date, makes it a promising candidate for such therapies. Further bioengineering of the nisin peptide could also provide additional information to further enhance its activity or characterise structure function relationships.

1.2 Introduction

Bacteriocins are antimicrobial peptides produced by both Gram-positive and Gram-negative bacteria. They can have either narrow (select bacterial types such as Gram-negative or Gram-positive) or broad (wider range of bacteria) spectrum antimicrobial activity. Many bacteriocins, such as the lantibiotics nisin and lacticin 3147, are produced by the food-grade lactic acid bacteria (LAB), *Lactococcus lactis* (*L. lactis*)[1,2]. Lantibiotics are ribosomally synthesised antimicrobial peptides that are subject to post-translational modifications resulting in the formation of unusual amino acids including lanthionine and β-methyllanthionine bridges. These unusual amino acids are created enzymatically when a covalent bridge is formed between a cysteine (C) residue and a dehydrated serine (S) (dehydroalanine, Dha), or threonine (T) (dehydrobutyrine, Dhb)[2]. Nisin and lacticin 3147 are two of the most extensively characterised lantibiotics to date. Both exhibit a broad-spectrum of antimicrobial activity at nanomolar concentrations by means of cell wall biosynthesis inhibition and pore formation[3–6]. Bacteriocins have a number of advantages which give them a wide-ranging potential to be used as future biological therapeutics such as their amenability to bioengineering, having an immunomodulatory role, synergistic activity with other antimicrobials, selective toxicity towards cancer cells, antimicrobial activity against numerous antibiotic resistant bacteria, low occurrence of resistance, low toxicity in humans, and high stability, solubility and activity[7–9]. However, bacteriocins are also known to have a relatively short half-life within the body due to certain digestive enzymes (such as trypsin and chymotrypsin)[10,11].

1.3 Lacticin 3147

Lacticin 3147 is a two peptide lantibiotic comprised of Ltnα which binds to the peptidoglycan precursor lipid II on the outer leaflet of the cytoplasmic membrane, and Ltnβ, which binds the Ltnα-lipid II complex and subsequently inhibits peptidoglycan synthesis and induces the formation of depolarising pores in the membrane of target cells[3]. It has displayed antimicrobial activity against numerous clinically significant pathogens, such as vancomycin-resistance enterococci (VRE), *Cutibacterium acnes* (*C.* acnes) (formerly known as *Propionibacterium acnes*), *Staphylococcus aureus* (*S. aureus*), penicillin-resistant *Streptococcus mutans* and *Mycobacterium tuberculosis*, along with synergistic activity with other antimicrobials (such as polymyxin B) against both Gram-positive and Gram-negative strains (methicillin resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli* respectively)s[12–16].

1.4 Nisin

This 34 amino acid peptide consists of 5 lanthionine rings, is cationic with 5 positively charged residues (Lysine (K) 12, K22, Histidine (H) 27, H31 and K34) and no negatively charged residues[17–19]. Like lacticin, nisin also interacts with lipid II resulting in cell wall biosynthesis inhibition and pore formation. Nisin A is an FDA and WHO approved GRAS (generally regarded as safe) peptide that has been used within the food industry as a preservative since 1953[7]. In addition, many studies have demonstrated the antimicrobial activity of nisin against clinically and veterinary significant bacteria such as MRSA, *Enterococcus faecalis* and *Streptococcus suis*, as well as displaying synergy with antimicrobials such as ramoplanin, chloramphenicol and β-lactams[9]. Since its discovery in 1928 nisin has been used in the food-industry as an alternative biopreservative, however in more recent years the potential applications of nisin within

the biomedical industry has expanded enormously[8,20–23]. Its role as an alternative therapeutic for various bacterial infections and cancers are just some of the possible avenues being investigated.

1.4.1 Nisin variants

To date eight natural nisin variants have been identified. Nisin A, Z, F and Q are produced by lactococci, variants U and U2 by *Streptococcus uberis*, nisin H by *Streptococcus hyointestinalis*, whereas nisin P is produced by both *Streptococcus gallolyticus* sbsp. *pasteurianus* and *Streptococcus suis*[24]. Nisin Z and A are closest in structure with only a single amino acid residue difference at position 27 (N and H, respectively)[25]. Nisin Z demonstrates a higher rate of diffusion and solubility, under neutral pH conditions, and has been isolated from several *L. lactis* strains[26,27].

One of the major advantages of lantibiotics over many traditional antibiotics is their gene-encoded nature. This makes them amenable to bioengineering, thus allowing for variants with additional advantageous properties to be developed. In addition to developing many variants with enhanced antimicrobial activity, stability and pharmacokinetic properties, bioengineering techniques have aided in the identification of peptide structure and function. Mersacidin and nukacin are both examples of bacteriocins that, after having undergone site-saturated mutagenesis, resulted in derivatives with enhanced bioactivity against clinically significant bacteria, such as MRSA, VRE and *Streptococcus pneumonia*[28,29].

Many nisin variants have been generated that demonstrate enhanced antimicrobial activity. In particular variants with targeted changes to the flexible hinge region at positions N20-M21-K22 exhibit enhanced activity against a host of clinically significant bacteria. Initially, two nisin Z mutants were generated, N20K and M21K, displaying enhanced activity against the Gram-negative bacteria *Shigella, Pseudomonas* and

Salmonella spp. and had a higher solubility when compared to wild type nisin Z[30]. This led to further studies involving the mutagenesis of the hinge region. Notably M21V displayed enhanced activity against a multitude of clinically significant bacteria, namely heterogenous vancomycin intermediate *Staphylococcus aureus* (hVISA), VRE, MRSA, *Clostridium difficile (C. dificile*), *Streptococcus agalactiae* (*S. agalactiae*) and *Listeria monocytogenes*[31,32]. Multiple variants with amino acid substitutions at position 29 have been shown to exhibit enhanced bioactivity against a range of drug resistant clinical, veterinary and food pathogens. Of those that were generated, two produced activity against both Gram-positive and Gram-negative pathogens, S29G and S29A[33]. Another study demonstrated that the bioactivity of nisin can be altered by changing the length of the hinge region[34]. Further studies involving the hinge region lead to the rational design of multiple variants (N20A-M21A-K22A (nisin-AAA) and N21S-M21A-K22A) exhibiting enhanced bioactivity against *L. lactis* HP, *S. agalactiae* ATCC 13813, *Mycobacterium smegmatis* MC2155 and *S. aureus* RF122[35]. This nisin-AAA variant was the subject of further bioengineering which resulted in the generation of a trypsin and chymotrypsin resistant variants[10]. Nisin is naturally inactivated by pancreatic and other proteolytic digestive enzymes such as trypsin and chymotrypsin upon ingestion, thus making it vulnerable to proteolytic breakdown in the gastrointestinal tract[10,11]. Trypsin and chymotrypsin cleavage sites in nisin A are located at positions 12, 20, 21, 22 and 31. By changing the amino acid residues at positions 12 and H31 of the nisin-AAA variant, trypsin and chymotrypsin cleavage sites were removed, allowing for potential survival upon entering the gastrointestinal tract[10].

1.5 Bacteriocin-antimicrobial synergistic and additive effects

With the incidence of antibiotic resistance on the rise, there is an urgent need for alternative therapies. The discovery of synergistic activity between bacteriocins and other antimicrobials may play a role in advancing the treatment of clinically significant pathogens[13,36–40]. With little evidence of resistance to lantibiotics, the combination of these antimicrobial peptides with classical antibiotics may help to reduce/overcome the emergence of resistance to the latter. With antimicrobials having varying levels of activity against different bacteria, combinations may allow for a broader range of pathogens to be targeted, including infections with unknown aetiology[9]. Furthermore, combining bacteriocins with other antimicrobial agents would allow for a decrease in effective dose and therefore could reduce adverse side-effects associated with certain antimicrobials[9,41]. Antibiotics, although frequently critical for patient recovery, can result in serious adverse side-effects. Polymyxin's are strongly associated with neurotoxicity, nephrotoxicity and renal toxicity (affecting 6 to54% of all patients), and vancomycin, an antibiotic widely used for the treatment of MRSA, is commonly associated with nephrotoxicity[42,43]. Using antibiotics, known to have toxic effects on the body, in combination with bacteriocins may help to reduce the level of toxicity.

1.5.1 Lacticin 3147

To date, lacticin 3147 has not been reported to exhibit any antimicrobial activity against Gram-negative bacteria. However, in combination with Gram-negative targeting antibiotics polymyxin B and polymyxin E (colistin), synergistic antimicrobial activity against the *Cronobacter sakazakii* DPC6440 and *E. coli* was clearly demonstrated[13]. The same study also showed synergy against Gram-positive *S. aureus* 5247, and *B. cereus* 8079 when lacticin 3147 was used in combination with polymyxin B[13]

1.5.2 Nisin

Multiple studies have shown the benefits of combining nisin with antimicrobial agents. For example, nisin has demonstrated synergy with many conventional antibiotics against clinically significant strains, such ramoplanin (also targets lipid II) against MRSA and VRE, penicillin and chloramphenicol against *E. faecalis*, polymyxcins against *Listeria innocua* HPB_{13}, *Acinetobacter* spp. and *E. coli*, and ciprofloxacin against MRSA and Methicillin sensitive *Staphylococcus aureus* (MSSA) strains[36,37,44–47]. Nisin has also demonstrated synergy with antibiotics such as vancomycin against MRSA strains, however the effect is not as dramatic, possibly due to their similar mode of action of binding to lipid II[47]. In the case of nisin-vancomycin, the binding of one antimicrobial to lipid II decreases the accessibility of lipid II to the second antimicrobial, thus combining antimicrobials that interact with different pathways are thought to elicit better synergistic activity[45,48]. In addition, nisin has also demonstrated antagonistic activity in combination with some antibiotics, for example nisin-chloramphenicol combinations against multiple MRSA strains[44].

Like lacticin 3147, the activity of nisin against Gram-negatives is poor but can be enhanced when used in combination with other antimicrobials such as polymyxin B and E, which have also proven to act synergistically with nisin against *C. sakazakii*, *Listeria*, *E. coli* and *Pseudomonas aeruginosa*[13,33,49]. Synergistic activity against *S. aureus* biofilm populations has also been demonstrated in multiple studies involving nisin and antimicrobial agents such as ciprofloxacin, daptomycin and lysostaphin[50,51]. Variants of nisin, such as M21V and I4V, have also proved to be effective in combination with antibiotics such as penicillin and chloramphenicol against planktonic and biofilm populations of *S. aureus* SA113 and *Staphylococcus pseudintermedius* DSM21284[52].

Antimicrobial agents other than antibiotics have also proven to work synergistically with nisin against an array of bacteria, including biofilm populations. Both sodium fluoride and polylysine work synergistically with nisin against biofilm and planktonic forms of *S. mutans*, as well as lactoferrin (secreted in the respiratory tract) working synergistically with nisin against *L. monocytogenes* and *E. coli* 0157:H7[8,38,53,54]. In addition, nisin has displayed synergistic activity when used in combination with ethylenediaminetetraacetate (EDTA) against *L. monocytogenes* and enterohaemorrhagic *E. coli* strains[55].

1.6 Nisin Induction

1.6.1 Nisin biosynthesis

The successful production of the mature nisin peptide is reliant on the inter-communication of several proteins and genes, and the mature peptide plays a role in its own induction through the specific activation of these genes[2,56–58]. Biosynthesis of nisin is encoded by a cluster of eleven genes (A, B, T, C, I, P, R, K, F, E and G). The first gene, *nisA*, encodes for the nisin precursor with the remaining genes coding for proteins responsible for peptide modification, translocation and processing (*nisB*, *nisC*, *nisP* and *nisT*) the bacteria's immunity against nisin itself (*nisI*, *nisF*, *nisE* and *nisG*) and regulating expression (*nisR* and *nisK*)[59].

The transmembrane histidine protein kinase NisK detects and binds to extracellular nisin before autophosphorylation. The subsequent phosphate group is transferred to, and activates, the intracellular response regulator NisR and triggers the activation of two promoters (P_{nisA} and P_{nisF}) on the nisin operon (Figure 1.1)[2,56–58,60]. This signalling by extracellular nisin to induce its own intracellular transcription is known as auto-induction.

To form the mature peptide, a precursor form of nisin with an N-terminal leader is first translated before being post-translationally modified. At this stage, selected serine (S) and threonine (T) amino acids are dehydrated by NisB and lanthionine bridges are formed by NisC. The peptide is subsequently exported out of the cell by NisT and cleaved of its N-terminal leader by NisP. The resulting peptide is fully mature and can now interact with NisK resulting in an auto-induction loop (Figure 1.1)[2,56–58].

Studies manipulating this process have shown that deletions in the structural *nisA* gene results in the termination of transcription, but this can be restored by the introduction of extracellular nisin peptide (and nisin variants) at sub-inhibitory levels. However when the *nisK* gene is disrupted the addition of extracellular nisin can no longer initiate the auto-induction loop[2]. One study showed that nisin can promote its own induction from within the cell itself[58]. The study concluded that the partial interaction of nisin with the signal recognition domain of nisK, within the water-soluble side of the membrane, was sufficient to induce a nisin auto-induction loop[58].

1.6.2 Nisin-controlled gene expression system

The advantages of using this nisin auto-induction mechanism, termed the Nisin-Controlled gene Expression (NICE) system, has been recognised and exploited for many years[59,61]. In recent decades, the NICE system has been used in the expression of numerous genes from diverse bacterial backgrounds and various applications, such as analysis of metabolic and enzyme function, and the production of greater quantities of protein for food, medical or technical applications[52,59]. Initially the *nisKR* genes, involved in signal transduction, were isolated and transferred into the *pepN* gene on the chromosome of *L. lactis* subsp. *cremoris* MG1363, a naturally plasmid and nisin free strain. This became known as NZ9000 and is the most commonly used host[57,59,62]. A gene of interest can be placed behind the inducible promoter P_{nisA} on a plasmid or the

chromosome and, so long as NisRK are present, will be expressed when induced by the addition of sub-inhibitory amounts of nisin (0.1-5ng/ml)[59].

1.7 Antibiotic Resistance

1.7.1 Global Significance

Antibiotic resistance has become a global problem, so much so that governing bodies have been compelled to act. In 2015 the WHO (World Health Organisation) issued a "global action plan on antimicrobial resistance"[63]. After the United Nations general assembly in 2016 all members were required to implement their own national action plan for combating antibiotic-resistant bacteria by 2017. In 2015 the United States Federal Government put in place the "national action plan for combating antibiotic-resistant bacteria" and in 2017 the EU Commission began implementing the "EU one health action plan against antimicrobial resistance (AMR)"[64].

Both reports highlight the importance of slowing the emergence of resistance bacteria, preventing the spread of resistant infections, accelerating basic and applied research, innovation and the development of new antibiotics, other therapies and vaccines[63,65]. In 2015 the WHO launched the *Global Antimicrobial Resistance Surveillance System* (GLASS) in an effort to support AMR research and the global action plan on antimicrobial resistance, and provide more information for the implementation of national, regional and global actions[66]. By 2017, 42 countries enrolled in GLASS, 40 of which provided information on their AMR surveillance systems and 22 of which provided actual AMR data[67].

1.7.2 Causation

Antibiotic resistance has become a global problem due to a multitude of factors, including: overuse, inappropriate prescribing, extensive agricultural use and the

availability of few new antibiotics[68]. It can occur through bacterial evolution, by spontaneous mutation or horizontal gene transfer (HGT). The overuse of antibiotics fuels the evolution of resistance, and allows for the accumulation of resistant bacteria and the passing of resistant genes through HGT, in some cases to very different bacteria[68].

1.8 Clinically significant antibiotic resistant bacteria

Antibiotic resistance remains a problem for numerous bacterial species with some giving a greater cause for global concern than others. The GLASS report of 2016-2017, closely monitored eight human bacterial pathogens, considered to be the greatest threat globally, from multiple countries worldwide[67]. The U.S. Centre for Disease Prevention and Control (CDC) and the European Centre for Disease Prevention and Control (ECDC) have released similar lists of pathogenic bacterial strains under observation (Tables 1, 2)[69,70].

1.8.1 Methicillin-resistant *Staphylococcus aureus* (MRSA)

MRSA is one of the top globally significant antibiotic resistant pathogens, it is monitored by both the CDC and the ECDC. *Staphylococcus aureus* is a Gram-positive coccus and is ubiquitous in nature with approximately 20-30% of individuals displaying nasal passage colonisation persistently and 60% intermediately[71–73]. It causes a multitude of skin infections ranging from superficial pimples, boils and impetigo, to deeper skin infections such as abscesses and cellulitis. MRSA is also frequently associated with wound infections, with surgical sites and intravenous lines identified as contributing risk factors[74–76]. In 2010 a study involving 33 medical centres in 13 European countries and Israel demonstrated that 71.1% of all skin and soft tissue infection (SSTI) isolates were *S. aureus*, 22.5% of isolates being methicillin resistant, and 9.3% were enterococci, 5.1% being VRE[77].

In the 1940s *S. aureus* infections were originally treated with penicillin and later (1960s) penicillin-related antibiotics such as methicillin and oxacillin. However, new forms of the pathogen, resistant to β-lactam antibiotics (including penicillins, cephalosporins and carbapenams), began to emerge shortly thereafter[78–80]. Reports have shown more recent cases of resistance to other antibiotics such as erythromycin, fluoroquinolones, tetracycline and clydamicin[81–83]. Vancomycin is now the drug of choice to treat an MRSA infection, but a decreased susceptibility to this drug is also on the rise. This form is known as vancomycin resistant *Staphylococcus aureus (VRSA)*, with an Minimum Inhibitory Concentration (MIC) value of ≥32μg/ml rendering it completely resistant[83]. Due to its highly mutable nature the threat of pandrug-resistance (resistance to all current drugs) is of growing concern for MRSA, with 95% of strains no longer responding to first line antibiotics and increasing numbers of studies reporting resistance to alternatives such as vancomycin[84]. In recent years the percentage of invasive MRSA isolates has decreased in Europe, however it remains a public health priority with a third of European countries reporting MRSA percentages above 25% (Figure 1.2).

1.8.2 Enterococcus faecium

E. faecium has been listed by the WHO as a global high priority pathogen and mentioned in the ECDC 2016 AMR surveillance report, with between 25 and 50% of invasive isolates being resistant to vancomycin in the Republic of Ireland, Poland, Latvia, Slovakia, Romania, Greece and Cyprus (Figure 1.3)[70]. In the years 2013-2016 the percentage of vancomycin-resistant *E. faecium* isolated were reported to have significantly increased in 7 of the 25 EU/EEA countries with a reported >20 isolates per year[70]. Vancomycin remains one of the first line treatments for Enterococcal infections. With increasing levels of VRE strains, new and effective treatments are required. *E. faecium* has also shown high levels of resistance to aminoglycoside antibiotics such as

gentamicin and can be intrinsically resistant to a variety of antibiotics such as clindamycin, ampicillin, penicillin and cephalosporins, making their treatment more difficult[85]. Due to its resistance to a variety of antibiotics, and its easy dissemination within healthcare settings, *E. faecium* is increasingly difficult to treat and has become a major pathogen of concern[70].

1.8.3 Cutibacteriumutibacterium acnes

Cutibacterium acnes, previously classed as *Propionibacterium acnes*, is a Gram-positive, aerotolerant, commensal human skin bacterium that contributes to the development of acne[86]. Although not as urgent a threat as MRSA and VRE, *C. acnes* affects 80% to 90% of the population at some stage in their life, and is the primary reason for dermatologist visits[87,88]. *C. acnes* is a normal part of the healthy skin flora, however it is an opportunistic pathogen contributing to the skin disease acnes vulgaris.

Recent studies have shown that the increased proliferation of *C. acnes* itself may not be the direct cause of acne, but rather the loss of skin microbial diversity and the activation of innate immunity triggered by the colonisation of *C. acnes* within the pilosebaceous follicle results in the subsequent development of acne[89]. Other studies reporting the increasing emergence of *C. acnes* resistant to various antibiotics, including fluoroquinolones, macrolides, erythromycin, clindamycin, tetracycline and trimethoprim-sulfamethoxazole have made this pathogen a cause for concern[90–92]. To date *C. acnes* remains susceptible to β-lactam antibiotics, including penicillin. However, the development of resistance either through the alteration of penicillin binding proteins (PBPs) or horizontal gene transfer remains a possibility. In a recent study a strain of *C. acnes* containing the β-lactamase gene was identified, which is not normally associated with Gram-positive bacteria, suggesting that it may be possible for the strain to acquired resistant to β-lactam antibiotics[93]. Consistent use of antibiotics, such as penicillin, also

increases the risk of other skin species developing resistance, exacerbating the overall antibiotic resistance crisis[94].

1.8.4 Corynebacterium xerosis

C. xerosis is a commensal bacteria found on human skin and mucous membranes and rarely causes infections of any clinical relevance, however there have been reports of the bacteria causing endocarditis in immunocompromised patients, postoperative infections and it also contributes to strong underarm odours[95–98]. Previous studies have shown that *C. xerosis* is growing in resistance to various antibiotics including β-lactams, aminoglycosides, macrolides, ciprofloxacin, chloramphenicol and tetracycline[98,99]. One study reports the finding of a multiply resistant strain shown to be the cause of mediastinitis (inflammation of the chest cavity) in a 69-year old asthmatic patient[98].

1.9 Bacteriocins as alternative therapeutics for bacterial infections

With the rapid evolution of resistance a growing number of skin and soft tissue infections are becoming increasingly difficult to prevent and/or treat, which has prompted research into viable alternative therapeutics, such as bacteriocins[8]. Bacteriocins have many benefits including low toxicity, amenability to bioengineering, low level of resistance occurrence, broad-spectrum antimicrobial activity, and have exhibited high levels of potency *in vitro* and significant activity against pathogens *in vivo*, suggesting that they may provide a possible alternative to antibiotics[23]. In one study the natural nisin variant, nisin F, demonstrated antimicrobial activity against *S. aureus* within the respiratory tract of rats when administered intranasally[100]. Another study exhibited the *in vivo* activity of the lantibiotic mutacin B-Ny266, against *S .aureus* in a mouse model of intraperitoneal infection and had a median effective dose (ED50) comparable to vancomycin[23,101].

Some bacteriocins have a broad-spectrum of activity, but their overall impact on the composition of the human microbiota is still unclear. Conventional antibiotics with broad spectrum ranges have been shown to cause damage to commensal populations which can be important for human health[102]. Numerous bacteriocins have demonstrated a broad-spectrum of activity, but one bacteriocin, Thuricin CD, has shown promising narrow-spectrum results against *C. difficile*. Post-treatment with thuricin CD the commensal microbiota in a human distal colon model was maintained, which suggests that there may be advantages to using narrow-spectrum bacteriocins[103,104].

The mechanism of antimicrobial activity varies between bacteriocins. Knowing the specific mechanism of action for a bacteriocin is an advantage when determining possible targets and conducting combination studies. As discussed previously, class I bacteriocins, such as nisin, act by targeting lipid II of Gram-positive targets and inhibiting peptidoglycan synthesis thus resulting in pore formation. However, other bacteriocins such as lactococcin A have shown activity against Gram-positive targets by binding to the pore-forming receptor mannose-phosphotransferase system (Man-PTS), along with others demonstrating activity against Gram-negative targets by interfering with DNA, RNA and protein metabolism (Figure 1.4)[23].

Bacteriocins have already demonstrated their therapeutic potential in a commercial setting in animals, with nisin being the active agent in the mastitis prevention product Wipe Out (ImmuCell Corporation) and with thiostrepton in combination therapy ointments for the treatment of dermatological indications. It is clear that the use of bacteriocins in a clinical setting for humans is highly plausible[23]. Furthermore, as mentioned previously in the cases of nisin and lacticin, combining bacteriocins with other therapeutics may yield synergistic effects due to differences in mechanism of action[9].

1.10 Cancer

With over 200 types of cancer and approximately 14.1 million new cases reported globally each year, it is not surprising that cancer is one of the most extensively researched areas in medicine[105]. In 2018 cancer will be responsible for an estimated 9.6 million deaths globally. In recent years, the number of global diagnoses has steadily increased and is expected to rise to 23.6 million new cases by the year 2030[105]. This increase is due to a number of factors which include a globally aging population, obesity, unhealthy diet with low fruit and vegetable intake, UV exposure, alcohol use, sexually transmitted infections, tobacco smoking and environmental factors, among others[106–109]. Other factors such as HIV/AIDS, immune factors, and organ transplantation have been shown to increase the risk of developing cancer due to patients having a compromised immune system[110]. Some countries have seen a decrease the number of cancer related deaths. In the U.S.A. for example the number of cancer incidence and related deaths has decreased from the years 1992 -2015 by 17% and 25% respectively, with an overall survival rate of 66.9% in 2014[111]. According to the World Health Organisation (WHO), 1 in 6 mortalities are attributable to cancer, making it the second leading cause of death worldwide, with an estimate of 9.6 million deaths[109]. In addition, the global economic impact of cancer is also increasing. In 2010, the total annual economic cost was estimated at US$1.16 trillion[109].

Traditionally the methods used to treat cancer include chemotherapy, radiotherapy, chemo-radiotherapy and surgery. They are known to have a variety of side effects ranging in severity including, but not limited to, nausea/vomiting, hair loss, weight loss/gain, neutropenia, stomatitis, diarrhoea, and aggravated insomnia[112–114]. With this being said, emphasis is now on the development of therapeutics that are safe and effective with fewer side-effects.

1.11 Colorectal cancer

In 2018 colorectal cancer (CRC) is estimated to have the third highest incidence rate of all cancers, approximately 1.8 million cases, and the second highest mortality rate with 862,000 deaths (8.98% of all cancer related deaths) (Figure 1.5)[109]. Adenocarcinomas account for approximately 96% of all CRCs and affect the mucus forming cells that aid in the internal lubrication of the colon and rectum[115]. Most CRC develop from a polyp, a growth on the inner lining of the colon or rectum, but not all polyps become malignant. Although individuals with colorectal cancer may not always develop symptoms some of the most common include: bloody stool, persistent stomach pains and unexplained weight loss[116].

1.11.1 Causation and Risk Factors

It is estimated that in 2018 CRC incidence was more common in males than females worldwide, 1,026,215 and 823,303 respectively, although it has the second highest incident rate of any cancer in females and third in males[117]. Recent findings suggest that "Western" populations and countries experiencing a rapid societal and economic transition are most at risk due to changes in dietary, reproductive and hormonal factors[118]. In the U.S.A. alone the lifetime risk of developing colorectal cancer is approximately 4.5% for men and 4.2% for women[119]. However, with an aging population the risk of developing CRC increases with advancing age, with 90% of cases occurring in patients 50 years or older. A diet low in fruits and vegetables, excessive weight and lack of exercise are three strongly linked risk factors to CRC. Diets low in fruits, vegetables and fibre, and high in fat, alcohol and red meat, are known risk factors of CRC, with lack of regular exercise and a high Body Mass Index (BMI) contributing to the risk[120].

Other factors such as tobacco use, inflammatory bowel diseases, for example Crohn's disease, and having a personal or family history of CRC can also increase the incidence[120]. Genetic syndromes such as hereditary non-polyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) account for a minority of cases[121].

In patients with FAP, multiple polyps can develop in the colon and/or rectum and can become cancerous over time unless the colon is removed. FAP can take three variations; classic, attenuated or autosomal recessive FAP. In patients with classic FAP polyps can form as early as their teenage years and become cancerous under 40 years of age[121]. The onset of cancer in patients with an attenuated form of FAP is on average 55 years of age[121]. In the case of autosomal recessive FAP typically fewer polyps develop (less than a hundred, rather than hundreds or thousands) and is subsequently less detrimental.

HNPCC is a hereditary disorder that increases the risk of many cancers, but particularly colorectal. HNPCC develops when mutations occur within certain genes involved in DNA repair (MLH1, MSH2, MSH6, PMS2 or EPCAM), which can lead to uncontrolled cell growth, and later cancer[122]. However, not all individuals that have mutations within these genes develop cancer[122].

1.11.2 Treatment

The course of treatment for CRC depends greatly on the cancer stage at the time of diagnosis. There are a number of treatment options available including: surgery, radiofrequency ablation, cryosurgery, chemotherapy, radiation therapy and immunotherapy[123]. Surgery is the most common treatment at any stage of CRC. A local excision surgery can be used for smaller early stage cancers, and resection by either a full or partial colectomy (removal of the cancerous tissue along with a small amount of surrounding healthy tissue) for larger tumours[123]. Due to a high recurrence rate, 30-40%,

adjuvant chemotherapy or radiation therapy are often used post operation to eliminate any remaining cancer along with reducing the risk of recurrence[124]. In rectal cancers, depending on the tissue type and stage of disease at diagnosis, a complete removal of the mesorectum is standard surgical procedure, followed by adjuvant chemotherapy and radiotherapy[125].

Chemotherapy drugs can be administered systemically (taken orally or injected into a vein or muscle to reach the blood stream) or regionally (administered directly into cerebrospinal fluid, organ or body cavity). Common chemotherapy drugs used to treat colorectal cancer include: 5-fluorouracil (5-FU), oxaliplatin and leucovorin (LV). 5-FU metabolises 5-fluoro-2'-deoxyuridine-5'-monophosphate, which inhibits the enzyme thymidylate synthase, depleting thymidine triphosphate and resulting in the termination of DNA synthesis. It also incorporates itself into RNA, where it inhibits RNA processing and subsequently cell growth, making it the primary therapy used for metastatic CRC (mCRC)[126,127]. Combination studies using 5-FU and multiple other chemotherapy drugs have shown great success. With overall response rates to 5-FU in late stages of CRC of 10-15%, combination treatments are employed using oxaliplatin which demonstrate improved effectiveness, but at the cost of increased toxicity[128]. Treatment with adjuvant 5-FU improves survival, demonstrating better results in stage III than in stage II disease, and is routinely used in combination with oxaliplatin for stage III disease, although efficacy versus toxicity levels must be closely monitored[129]. When 5-FU and oxaliplatin are used in combination, treatment time can be reduced, in some cases from 6 months to 3 months depending on the individual, which can aid in the control of toxicity levels[130]. For individuals who have recurrent colorectal cancer; local or distant, surgery and chemotherapy are often the treatment of choice. In addition, chemotherapy can also be used to palliatively treat metastatic cancer, to improve survival, lessen symptoms and

improve overall quality of life. 5-FU is commonly used with oxaliplatin and irinotecan as a palliative therapy and can increase survival by almost 3 years[127,129].

Immunotherapy involves components of the immune system such as antibodies, cytokines and dendritic cells, along with vaccines to kill tumour cells. Immunotherapy provides a form of therapy that specifically targets tumour cells[131,132]. Unlike other forms of cancer therapy, immunotherapy has not yet provided major advances in CRC treatment as it has for other forms of cancer, however some therapies have shown promise[132]. Pembrolizumab, a monoclonal antibody (mAb) has demonstrated a significant effect against CRC tumours which resulted in it being approved by the FDA as a treatment for CRC[132]. The FDA has also approved the use of multiple monoclonal antibodies for use in colon cancer including, Bevacizumab (Avastin), Cetuximab (Erbitux) and Panitumumab (Vectibix). Bevacizumab, first introduced in clinical trials in 1997, is a relatively non-toxic monoclonal antibody drug that targets vascular endothelial growth factor (VEGF)[127]. VEGF is involved in controlling angiogenesis, the growth of blood vessels which in turn allows for proliferation of tumour cells, and high levels are associated with metastatic disease[127]. Bevacizumab is an angiogenesis inhibitor and acts by binding and neutralising VEGF. Bevacizumab has also been used in combination with specific chemotherapy regimens, for example 5-FU/LV, and has demonstrated significant improvement in the median survival and disease progression times of mCRC patients[127,133]. However, the use of bevacizumab has been associated with perforation of the bowel, in approximately 1.7% of patients[134]. Cetuximab and Panitumumab both target epidermal growth factor receptor (EGFR), a protein over expressed in 60%-80% of tumours and associated with tumour growth[135].

1.11.3 Survival

Although the survival rate has increased in recent decades, CRC remains the third most commonly diagnosed cancer in men and second in women[109,119,125,136]. The prognosis for a patient can range from a five year survival rate as high as 92% to as low as 10%, and is highly dependent on the stage of disease at diagnosis[137]. Typically, CRC has a 5-year survival rate of 90% when detected at the localised stage, 70% for regional, to 10% for distant mCRC[137]. As with most cancers, the earlier the diagnosis, the higher the chance of survival. Age also plays a role, with younger individuals generally displaying better survival rates. The 5-year survival rate for younger patients (15-45 years of age) can be as much as 15% higher than that for older patients[138].

In the USA CRC mortality rates have declined since 1970 by approximately 52% among both men and women, which is most likely attributed to improved treatments and increased screening. However, mortality rates of individuals less than 55 years of age increased by approximately 9% from 2006 to 2015[139]. Without treatment patients with advanced CRC have a median survival time of 5-6 months, and with 5-FU chemotherapy based treatment approximately 10-12 months, with less than 5% surviving beyond 5 years[138]. In the European Union member states CRC mortality fell by approximately 13% in men and 27% in women from the years 1989 to 2011 compared to 39.8% and 38.8% in the U.S.A.[140]. In contrast, there have been rapid increases in in CRC incidence and mortality rates in many eastern European countries[125].

1.12 Oesophageal cancer

OC is a notoriously aggressive and invasive form of cancer with 20-30% of patients exhibiting distant metastases at the time of initial diagnosis[141]. To date oesophageal cancer (OC) globally has the seventh highest incidence rate of all cancers,

approximately 572,034 cases, and the sixth highest mortality rate with 508,585 (5.3%) deaths (Figure 1.5)[109]. OC incidence is more common in males than females worldwide (399,699 and 172,335, respectively) with the vast majority of OCs being either squamous cell carcinomas (SCC) or adenocarcinomas (AC)[117]. Both forms are extremely aggressive and associated with high mortality rates[142]. SSC affects the cells comprising the inner lining of the oesophagus and tend to develop in the upper and middle section of the oesophagus, and is the most prevalent form of OC worldwide[143]. ACs develops in gland cells, which in the case of OC are involved in producing mucus in the lining of the oesophagus, and are typically associated with the lower part of the oesophagus Adenocarcinomas are the most common type of OC reported in the U.S.A.[143,144]. Incidences of OC have been increasing steadily since the 1960s/1970s and are expected to increase even further in the future. In 2014 it was estimated that by the year 2025 the prevalence of OC will increase by 140%[141].

As with other forms of cancer, an aging population is a contributing factor to tumour development with 80% of oesophageal cancers developing in people aged 60 or older[145]. Although individuals with OC may not always develop symptoms, some of the most common include: dysphagia (difficulty swallowing), chest pain, coughing and unexplained weight loss.

1.12.1 Causation and Risk Factors

Many studies have reported alcohol as the primary risk factor for the development of oesophageal malignancy, along with the impact of tobacco use. Reports have also suggested that alcohol and smoking can act almost synergistically to increase the risk[141]. Alcohol is a known solvent of many of the hazardous carcinogens within tobacco, thus allowing them to penetrate the oesophagus epithelium easier[141]. It is also notable that

exposure to risk factors can be linked to geographical and racial differences, with an incidence rate of three times higher in black individuals than white[141,146].

Another major risk factor for OC is the presence of Barrett's oesophagus, a condition involving the replacement of normal flat oesophagus squamous cells to abnormal columnar cells (dysplasia). Up to 5% of all individuals with Barret's oesophagus go on to develop adenocarcinoma at some stage[147]. Males, especially Caucasian males, are 2-3 times more likely to develop Barrett's oesophagus than females, putting them at a higher risk of developing OC[148]. Acid reflux is a major cause of Barrett's oesophagus and can be exacerbated by smoking, consuming spicy or fatty foods, excessive alcohol intake and obesity[147]. Gastroesophageal reflux disease (GERD) has been shown to increase the risk of developing Barrett's oesophagus with individuals diagnosed with GERD being 6-8 times more likely to have Barrett's oesophagus[148,148].

Other risk factors include lack of fruits and vegetables in the diet, regularly consuming very hot liquids, and undergoing radiation treatment for other cancers to the chest or upper abdomen[145]. Tylosis with oesophageal cancer is a genetic condition characterised by non-epidermolytic palmoplantar keratoderma and a lifetime risk of developing oesophageal SSC of up to 95% by the age of 65[149]. It involves the thickening of the palms of the hands and soles of the feet but can also result in the thickening of the oral mucosa[149].

1.12.2 Treatment

Treatment strategies for oesophageal cancers are dependent on the tumour staging at the time of diagnosis. When surgical resection alone is used to manage local SSC and adenocarcinomas of the oesophagus, survival rates are generally low and metastatic recurrence levels are high[150,151151]. These patterns prompted the use of multimodal approaches. For locally advanced OC, surgery is generally used with curative intent for

patients suitable for resection, with neoadjuvant treatment strategies if required. Neoadjuvant therapies (preoperative) normally consist of either chemotherapy, radiation therapy or a combination of both, and are used primarily over adjuvant therapy (postoperative)[151]. The majority of patients receive neoadjuvant chemotherapy, with therapies often including oxaliplatin/5-FU regimes[141,152]. One study, using 5-FU and cisplatin as a neoadjuvant chemotherapy regime, resulted in an improved 2-year survival rate, when compared to surgery alone; 43% and 34% respectively[150]. The use of radiation therapy in combination with chemotherapy (chemoradiotherapy) at the preoperative stage is still controversial along with the use of adjuvant chemotherapy. Recent reports suggest the use of neoadjuvant chemotherapy alone and to reserve the use of adjuvant chemotherapy for rare and select patients[151]. Studies conducted by the Japan Clinical Oncology Group compared the results of three approaches; using surgery alone, neoadjuvant chemotherapy, and postoperative adjuvant chemotherapy in patients with SCC; using cisplatin and 5-FU chemotherapy regimes[153]. In these studies the overall 5-year survival did not differ significantly between patients who received postoperative chemotherapy compared with surgery alone (52% versus 61%), but disease-free survival was improved[153]. These studies also reported an increased 5-year survival rate when neoadjuvant chemotherapy was used in comparison to postoperative chemotherapy, 60% compared to 38%, respectively[153]. Other studies compare the use of chemoradiotherapy alone or as a neoadjuvant therapy. In one such study, involving oesophageal SSC, the 2-year survival rates were similar in neoadjuvant chemoradiotherapy (39.9%) and chemoradiotherapy (35.4%) patients[153].

However, each treatment modality was associated with its own set of side effects. Neoadjuvant chemoradiotherapy was commonly associated with increased levels of postoperative mortality whereas chemoradiotherapy alone had a higher rate of local

relapses[153]. However, numerous studies have shown the advantages of chemoradiotherapy, which remains the basic strategy for patients with locally advanced oesophageal SSC or adenocarcinoma[141,154]. Despite advances in these areas, locally advanced OC is still an aggressive malignancy with poor survival rates[152].

A promising new approach to OC treatment is the use of monoclonal antibodies. Certain mAbs have the ability to recognise and target abnormal proteins expressed by cancer cells and can influence how the cancer cell functions and flag these cells to the immune system. Vascular Endothelial Growth Factor (VEGF), Epidermal Growth Factor Receptor (EGFR) and Her-2 are common targets for these mAbs. EGFR is a transmembrane glycoprotein with tyrosine kinase activity, which when over expressed significantly increases cellular proliferation, induces angiogenesis and the development of metastasis, and inhibits the apoptotic pathway[155]. Studies report it to be over expressed in 50-70% of OCs[155].

Erlotinib is a monoclonal antibody that inhibits intracellular tyrosine kinase associated with EGFR, and induces cell cycle arrest and apoptosis. It has shown promising results in the treatment of unresectable or metastatic gastric or gastroesophageal cancers with reports of a 20% survival rate of 6.7 months[156].

Trastuzumab is a humanised monoclonal antibody that targets human epidermal growth factor receptor 2 (Her-2). Her-2 is over expressed in 0-56% of patients with oesophageal squamous cell carcinoma and 10-40% of patients with oesophageal adenocarcinoma and can lead to growth promotion in cancer cells[157,158]. Trastuzumab has already proven to effective against breast cancer and is sold under the name Herceptin®. In addition trastuzumab has exhibited promising results against oesophageal cancer cell lines, exhibiting a 50% reduction in Her-2 expression when compared to untreated cells, and a reduction in metastases in an *in vivo* model[157].

1.12.3 Survival

Oesophageal adenocarcinoma generally have a poor prognosis with a relative 5-year survival rate of <20%, whereas squamous cell carcinoma has a relative 5-year survival rate of ~12%[148,159]. Overall OC has a relative 5-year survival rate for localised disease of 43%, regional disease of 23% and distant disease of 5%[160].

More than 50% of patients have either unresectable tumours or detectable distant metastases at the time of diagnosis[161]. The survival rate of patients who do undergo resection varies greatly depending on the stage of cancer at the time of surgery. After complete surgical removal of the localised tumour, the 5-year survival rate can be as high as 95% for stage 0 patients, and as low as 10-15% for stage III patients[161]. Patients diagnosed at stage IV disease are not suitable for surgical treatment and are treated palliatively, with a median survival rate of <1 year[162].

1.13 Modes of cancer cell death

Currently over 34 different modes of cell death have been described[163]. A recent report categorised all 34 into four basic modes of cell death, two physiological; senescent death (death from cellular aging) and apoptosis, and two pathological; necrosis and stress-induced cell death[163]. However, other reports have classified them based on their morphological appearance (which can be either apoptotic, necrotic, autophagic or mitosis associated), enzymological criteria, functional aspects (programmed or accidental, physiological or pathological) or immunological characteristics[164]. Here we focus on apoptosis, and autophagic/type II cell death, a stress-induced cell death mechanism closely linked to apoptosis. Both exhibit very different morphological features and undergo different cell death pathways (Table 3).

1.13.1 Apoptosis

Apoptosis is a form of programmed cell death (PCD) that cells employ to maintain homeostasis. It is a normal occurrence during cell development and aging as a means to control cell populations, however it can also be used to eliminate cells that have become damaged due to infection, or as a defence mechanism during immune reactions[165]. Apoptosis can be activated by numerous stimuli and can also result from multiple pathways. Chemotherapy drugs can stimulate apoptosis by damaging cellular DNA, and depending on the stimulant, damaged cells can undergo different apoptotic pathways. A study using hepatocellular carcinoma (HCC) and a range of chemotherapeutic agents, demonstrated that certain drugs, such as bleomycin, can stimulate a *p53* dependent pathway, and subsequently either an intrinsic or extrinsic apoptosis signalling pathway[166]. At low doses anticancer treatments such as chemotherapy drugs or radiation can stimulate apoptosis, however, at higher concentrations they can also stimulate necrosis[165,167].

Apoptosis is morphologically distinguishable by the presence of pyknosis (condensation of chromatin in the nucleus) and extensive cytoplasmic blebbing among others characteristics (Table 3). During apoptosis, DNA is fragmented and the chromatin is dispersed throughout the cell before being packaged into apoptotic bodies along with cytoplasm and other organelles[165]. This process is known as "budding". *In vivo* these bodies are phagocytosed by immune cells, such as macrophages, and degraded[165].

The extrinsic and intrinsic pathways are among the most predominant apoptotic pathways taken by cells. Both pathways involve numerous enzymes known as caspases and are activated by the cleavage of caspase-3 (Figure 1.6). Upon stimulation, the activation of one caspase activates the next, resulting in what is known as a caspase cascade. This proteolytic mechanism amplifies the apoptotic pathway, resulting in rapid cell death[165]. There are 14 known caspases to date, with the ten major ones being generally

categorised as follows: initiator caspases (caspase 2, 8, 9, 10) effector caspases (caspase 3, 6, 7) and inflammatory caspases (1, 4, 5)[165]. Other apoptotic pathways have been identified, including multiple endoplasmic reticulum stress pathways where caspase cascades are activated, including caspases-12 (Figure 1.6). Each pathway requires the activation of either the initiator caspase -8 or -9, which subsequently results in the activation of the effector caspase, caspase-3.

Phosphatidylserine is displayed externally on apoptotic cells, which allows phagocytes to quickly recognise them for disposal and avoid an inflammatory response[165]. Annexin V is a phosphatidylserine-binding protein and can be used for the detection of apoptosis[165].

Upon initiation of the extrinsic pathway through the activation of transmembrane receptors, known as death ligands and receptors or death effector domain (some of the best characterised ligands and corresponding receptors include FasL/FasR and TNFα/TNFR1), the receptors recruit and bind to cytoplasmic adapter proteins (FADD and TRADD)[165,168]. These adapter proteins subsequently result in the activation of caspase-8 (Figure 1.6)[165].

Stimulants of the intrinsic pathway are non-receptor-mediated and produce intracellular signals (such as DNA damage and growth factor deprivation) that are mediated by the mitochondria[165]. Particular growth factors, hormones and cytokines are necessary for the suppression of certain apoptotic pathways, and in their absence, due to specific stimuli, apoptosis can be activated[165]. These are known as negative signals. Stimuli, such as radiation for example, can also generate positive signals that activate apoptosis[165]. Changes in the mitochondria, due to the stimuli, result in the release of pro-apoptotic proteins, including cytochrome *c*, from the mitochondrial permeability transition pore into the cytosol[165,169]. The release of cytochrome *c* is both positively and

negatively regulated by the Bcl-2 family[170]. Cytochrome *c* activates Apaf-1 (apoptotic protease activating factor 1), and procaspase-9, thus forming a large protein complex known as an apoptosome, which subsequently leads to the activation of caspase-9 (Figure 1.6)[165,170,171]. Other pro-apoptotic proteins are released from the mitochondria during apoptosis which are involved in DNA fragmentation and chromatin condensation, and are also regulated by members of the Bcl-2 family[165,172]. Tumour suppressor proteins such as *p53* have shown to play a role in the regulation of Bcl-2, an anti-apoptotic protein[173]. There is also evidence of "cross talk" between the extrinsic and intrinsic pathways, involving the cleavage of the pro-apoptotic protein BID by caspase-8, leading to mitochondrial damage and thus apoptosis[174]. Once caspase-3 has been activated by an initiator caspase (caspase-8, -9, or -10), it in turn activates CAD, an endonuclease that is responsible for fragmenting DNA[165,175].

1.13.2 Autophagic / Type II cell death

Typically, the role of autophagy is to promote cell survival under metabolic stress through the degradation of intracellular components and their subsequent recycling. Even if extracellular nutrients are plentiful, the deficiency in components such as growth factors can lead to the internalisation and degradation of nutrient transporters thus depriving the cell of nutrients[176]. The lack of extracellular nutrients and growth factor signalling stimulates apoptosis, however cells have the ability to activate autophagy in a bid to provide energy for mitochondrial activity and ultimately save the cell[176]. During autophagic activity, proteins, organelles and other cytoplasmic components are taken up into a double-membrane structure known as an autosome, which subsequently fuses with a lysosome to break down each component[177]. Furthermore, autophagic activity can be initiated to control the quality of cytoplasm by eliminating protein aggregates and damaged organelles[177].

Autophagy morphology is distinguished by large autophagic vacuoles in the cytoplasm and, in comparison to apoptosis, the absence of chromatin condensation (Table 3). In contrast to apoptotic cells, autophagic cells *in vivo* have little to no association with phagocytes[164].

In addition to autophagy acting as a survival mechanism it has been suggested as a mode of cell death[165,178]. Normally autophagy precedes apoptotic cell death however autophagic cell death has been known to occur in situations where cells were unable to activate apoptosis. One study showed that in the absence of the pro-apoptotic proteins, BAX and BAK (important for the release of specific proteins from the mitochondria during apoptosis), cell death was achieved by autophagy, which is regulated by Atg5 and Beclin1[179]. Interestingly, another study showed that Beclin1 is required for both Atg5 dependent and independent autophagy[180]. Additional studies, including an *in vivo* study, have been conducted showing the importance of autophagic activity for cell death, during *Drosophila* development[181,182]. Furthermore, reports have suggested certain cell death pathways are autophagy-dependent, however it is still controversial as to whether autophagy alone leads to cell death or facilitates other death pathways[177].

The role of autophagy in cancer is controversial. In general it functions as a tumour suppressor pathway by preventing tumour initiation but it can also act as a survival pathway taken by tumour cells to survive metabolic stress and in some cases resist death caused by certain cancer chemotherapy[183]. Indeed one commercial product that induces autophagic activity has been developed, tamoxifen, which exhibits preventative activity against breast cancers, however other agents involved in the suppression of autophagic activity are also undergoing clinical trials[183]. One study raises the concern of preventing autophagy-dependent anticancer immune responses and therefore limiting the impact of chemotherapy treatment[183,184]. Moreover, due to

autophagic activity aiding in tumour suppression, the initial benefits of suppressing tumour progression achieved by autophagy suppression, may lead to increased incidence of secondary malignancies[183].

1.14 Bacteriocins as alternative cancer therapeutics

As previously described, current cancer therapies have non-specific toxicity which affects healthy cells and causes undesirable side-effects[21]. Moreover, cancers have been known to develop resistance to certain chemotherapy drugs, accentuating the need for novel therapies[21]. In recent years several studies have begun to explore the potential of bacteriocins as alternative anti-cancer therapeutics[21]. These studies, have suggested that bacteriocins may be capable of selectively binding to cancer cells due to these cells exhibiting a different membrane fluidity to healthy cells, further accentuating their promise as future anti-cancer agents[20,21,185].

A number of bacteriocins have been investigated for anti-cancer activity including nisin, colocins, microcins, pyocins,pediocins, plantaricin A, bovicin and smegmatocin[21]. Varying levels of anti-cancer activity were demonstrated, mainly *in vitro*, against a wide range of cancer cell lines including human colorectal cell lines (HT29 cells)[186]. These studies are at the forefront of the exploration into the potential use of bacteriocins in cancer therapy. It is relevant to note that different bacteriocins will have varying effects on cancer cells, bactofencin for example has been shown to decrease cell proliferation of HT29 cells but increase cell proliferation of HCT116, Caco2, OE19 and SW620 cell lines (Figures A 1.2, A 1.3). The varying levels in activity suggest that more research should be done conducted as cytotoxicity appears to be bacteriocin and cell line dependent.

1.14.1 Nisin as an alternative cancer therapeutic

The interest in nisin over other bacteriocins is most likely due to its GRAS status and that it is one of the most highly researched bacteriocins to date[187]. It has been the subject of numerous studies in recent years with regards to its anti-cancer potential and has demonstrated cytotoxic activity against various cancer cells lines *in vitro* and *in vivo*[22,188]. Studies demonstrating nisin A's cytotoxicity against human colon cells (SV40-HC cells) were carried out in 2003, with percentage cell survival of <50% after 48hrs of treatment with all concentrations tested (170AU/ml, 350AU/ml and 700AU/ml)[189]. Further *in vitro* studies showed the cytotoxic effects of nisin A on two colorectal adenocarcinomas, HT29 and Caco2 24hrs post treatment, and one breast adenocarcinoma, MCF-7 48hrs post treatment, with IC_{50} (concentration at which half of the cells are inhibited) MTT values of 89.9μM, 115.0μM and 105.46μM, respectively[190,191]. Morphological changes in nisin A treated MCF-7 cells, when compared to untreated controls, were observed through microscopic examination including vacuolization of the cytoplasm, cell shrinkage, condensation and lateralisation of the nucleus which may suggest death by apoptosis but in the presence of autophagic activity[191].

A more recent *in vitro* and *in vivo* study investigated the cytotoxic and anti-tumour properties of nisin A against head and neck squamous cell carcinoma (HNSCC)[22]. The study demonstrated nisin A's ability to lessen HNSCC tumorigenesis via CHAC1, a proapoptotic cation transport regulator, and a contributing CHAC1-independent influx of extracellular calcium[22]. It has been suggested that the ability of nisin to differentially alter the transmembrane potential and membrane composition of HNSCC cells in comparison to the primary keratinocytes, may be due to differences in the structure and function of their lipid membrane and response to calcium influxes, and that this may be the reason for its preferential anti-cancer activity[8]. Apoptosis was induced along with reduced

HNSCC cell proliferation, and cell cycle arrest when treated with 80μg/ml nisin[22]. The study also included an *in vivo* oral xenograft mouse model whereby nisin significantly reduced tumorigenesis[22].

A later study was completed using nisin ZP, with a higher active content of 95%, for the treatment of HNSCC in both *in vitro* and *in vivo* mouse models[192]. The data supported the potential use of nisin as an alternative therapeutic by demonstrating an increased level of apoptosis, and decreased levels of cell proliferation, clonogenic capacity and sphere formation in HNSCC cells. In addition, long-term treatment with nisin ZP extended survival in mice with normal organ histology and no evidence of inflammation, fibrosis or necrosis[192].

The use of bacteriocins in conjunction with currently used anti-cancer drugs was also investigated in a recent study demonstrating the potential benefits of combining nisin with the anti-cancer drug cisplatin. Synergistic activity was observed between nisin and cisplatin when used to treat HNSCC cells that are normally highly resistant to cisplatin and ionizing radiation[188]. Furthermore, the addition of nisin was shown to increase the effectiveness of doxorubicin treatment, evident by the *in vivo* reduction of tumour severity in skin carcinogenesis[193].

1.15 Conclusions

The growing concern of antibiotic resistance highlights the need for alternative therapeutics. The assessment of different antimicrobials, such as bacteriocins, in therapeutic strategies, as sole agents or in combination, allows for the reassessment of how conventional antimicrobials are currently used in therapeutic regimes. With lantibiotics presenting a naturally low occurrence of antibiotic resistance these modified peptides may serve as an alternative to or could be used in conjunction with conventional antibiotics. In addition, bioengineering techniques have aided in the identification of the structure and function of various peptides, and to the creation of new variables with enhanced activity and stability. Further manipulation of such peptides could lead to future tailoring of genetic variants to help overcome issues such as resistance.

Cancers of the gastrointestinal system, particularly those of the oesophagus and colon, are known for their late detection and poor prognosis with therapeutic regimes often accompanied by multiple adverse effects. The use of bacteriocins may offer an alternative approach to anti-cancer treatments, having shown effectiveness against a variety of cancers *in vitro* and *in vivo* with little to no adverse effects.

In conclusion, bacteriocins may have multiple biomedical applications in the future and it seems timely to explore this potential.

Urgent threats	Serious threats	Concerning threats
• *Clostridium difficile* • Carbapenem-resistant Enterobacteriaceae (CRE) • Drug-resistant *Neisseria gonorrhoeae*	• Multidrug-resistant *Acinetobacter* • Drug-resistant *Campylobacter* • Fluconazole-resistant *Candida* • Extended spectrum beta-lactamase-producing Enterobacteriaceae (ESBLs) • Vancomycin-resistant Enterococci (VRE) • Multidrug-resistant *Pseudomonas aeruginosa* • Drug-resistant nontyphoidal *Salmonella* • Drug-resistant *Salmonella ttyphimurium* • Drug-resistant *Shigella* • Methicillin-resistant *Staphylococcus aureus* (MRSA) • Drug-resistant *Streptococcus pneumoniae* • Drug-resistant *tuberculosis*	• Erythromycin-resistant Group A *Streptococcus* • Clindamycin-resistant Group B *Streptococcus* • Vancomycin-resistant *Staphylococcus aureus* (VRSA)

Table 1.1 CDC Assessment of Antibacterial Resistance Threats in the U.S.[69].

Antibiotic resistant pathogenic bacteria	
• *Escherichia coli* • *Klebsiella pneumoniae* • *Pseudomonas aeruginosa* • *Acinetobacter species*	• *Streptococcus pneumoniae* • Methicillin-resistant *Staphylococcus aureus(MRSA)* • Enterococci (including *E. faecium* and *E. faecalis,*

Table 1.2 Antibiotic resistant pathogenic bacteria under surveillance in Europe[70].

Cell death mode	Morphological features
Apoptosis	Reduction of cellular and nuclear volume (pyknosis) Nuclear fragmentation (karyorrhexis) Minor modification of cytoplasmic organelles Plasma membrane blebbing Engulfment by resident phagocytes, *in vivo*
Autophagy	Lack of chromatin condensation Massive vacuolization of the cytoplasm Accumulation of (double-membraned) autophagic vacuoles Little or no uptake by phagocytic cells, *in vivo*
Necrosis	Cytoplasmic swelling (oncosis) Rupture of plasma membrane Swelling of cytoplasmic organelles Moderate chromatin condensation

Table 1.3 Distinct modalities of cell death – adapted from Kroemer *et al.*, 2009[164].

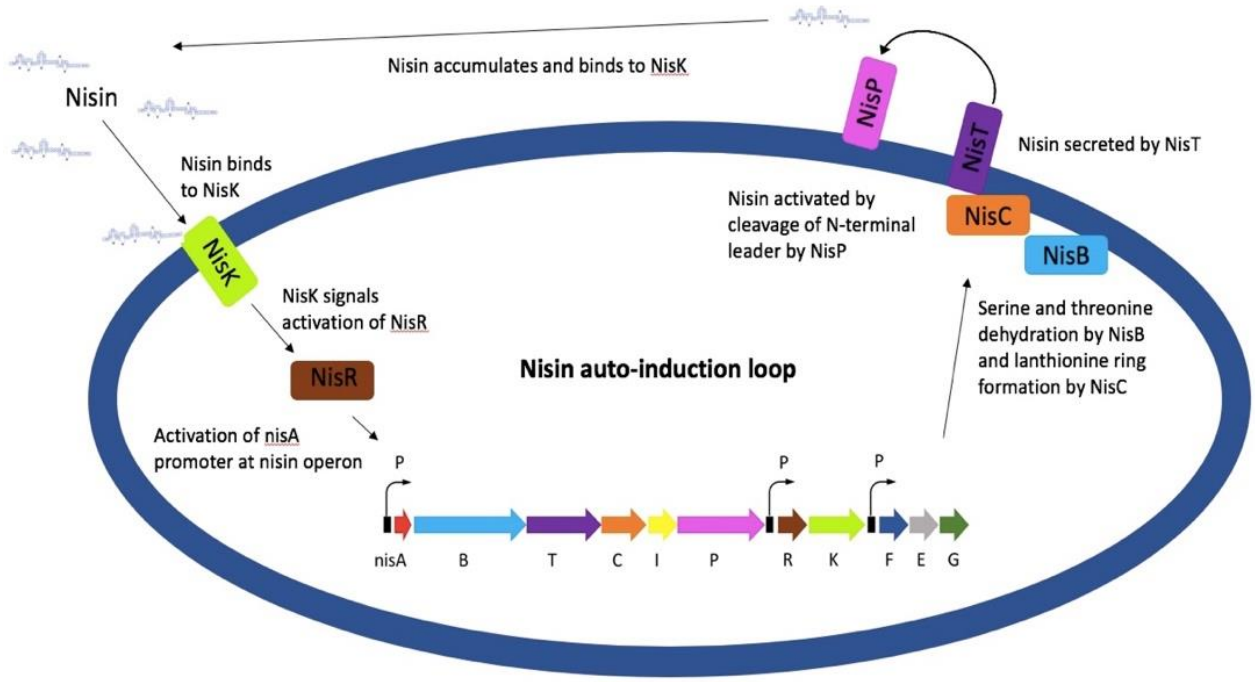


Figure 1.1 Nisin auto-induction loop schematic, adapted from Mierau et al., 2005.

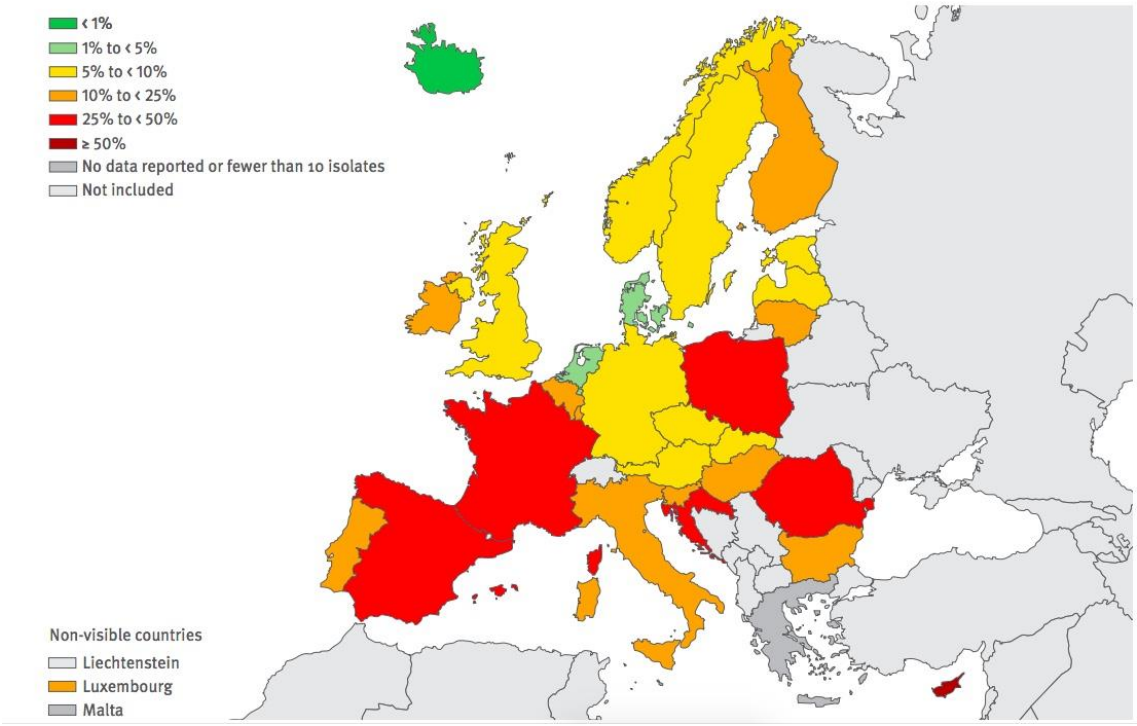


Figure 1.2 Percentage of invasive MRSA isolates in the EU/EEA, in 2016[70].

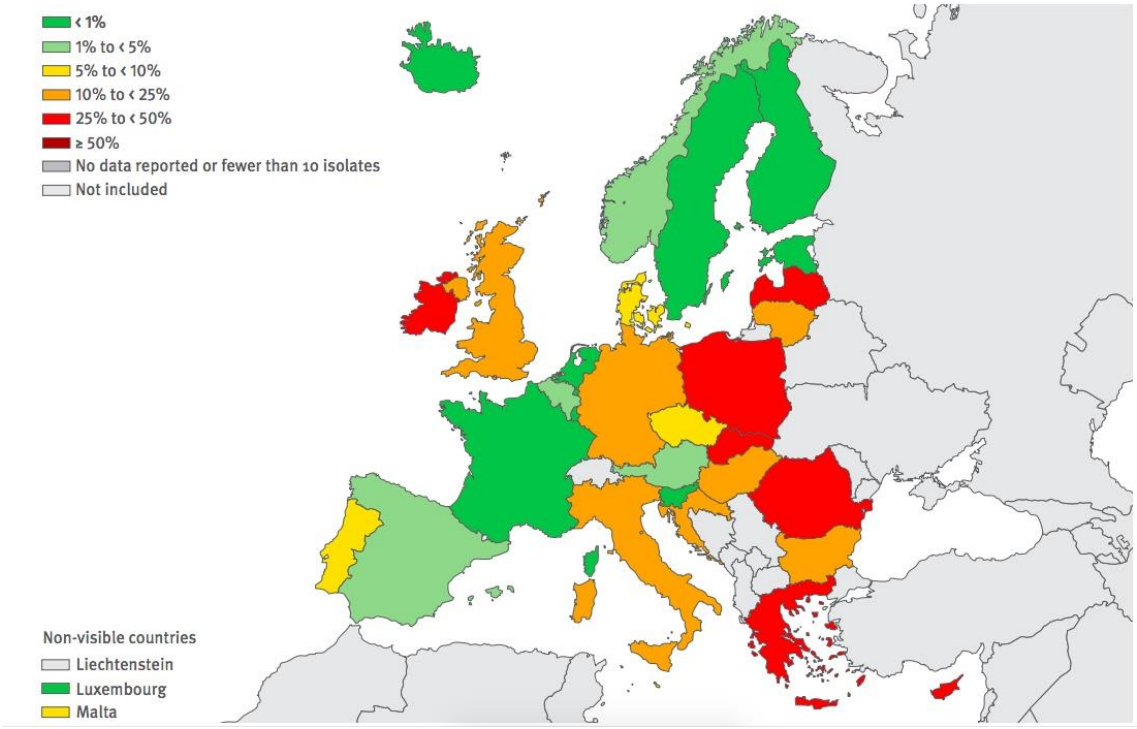


Figure 1.3 Percentage of invasive vancomycin resistant *E. faecium* isolates in the EU/EEA, in 2016[70].

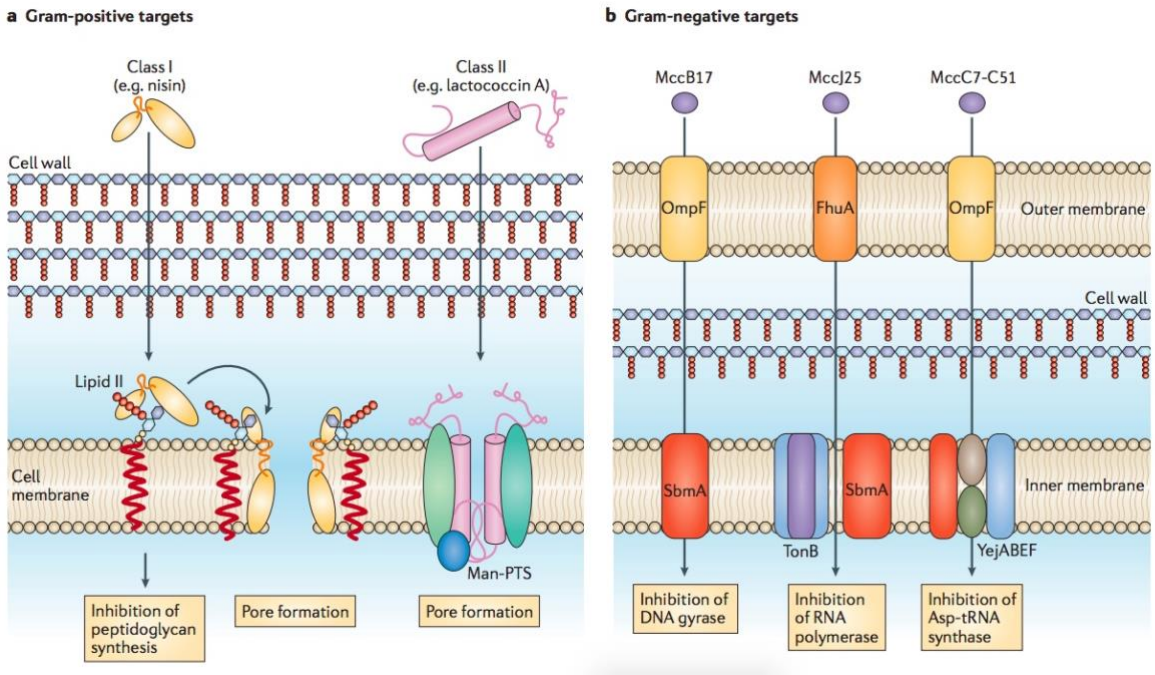


Figure 1.4 Mechanism of action of representative bacteriocins[23].

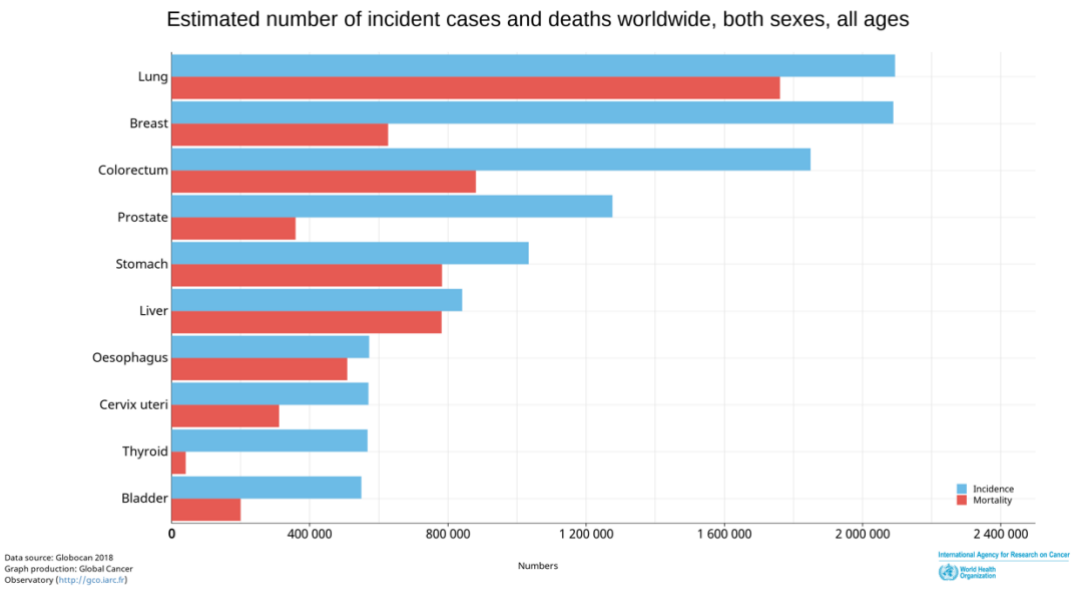


Figure 1.5 GLOBOCAN 2018 estimated total number of colorectal cancer incidences worldwide[117].

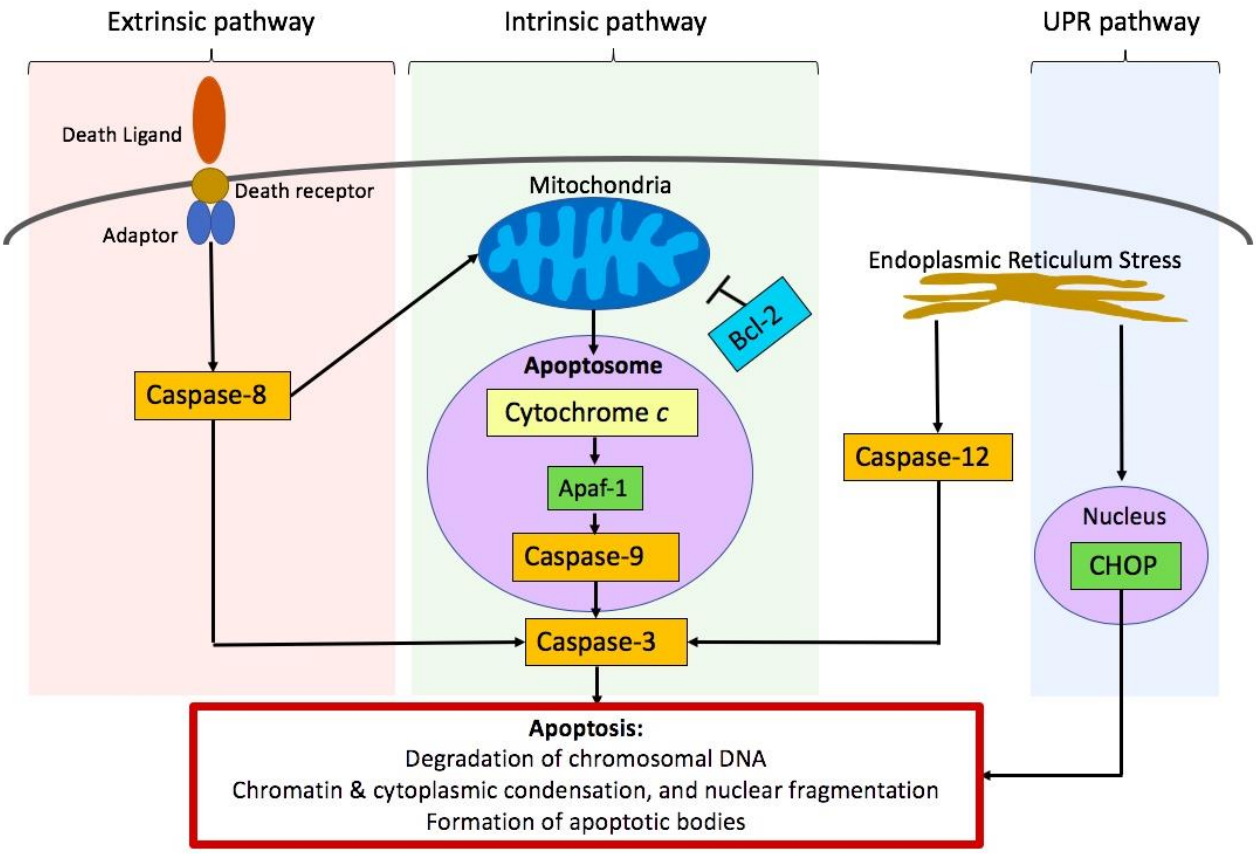


Figure 1.6 Apoptosis pathways adapted from John C. Reed and Maurizio Pellecchia, 2005, Oshitari T. *et al.*, 2008, Susan Elmore, 2007, and S. Fulda and K-M. Debatin, 2006[165,194–196].

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Chapter II

Analysis of nisin-AAA induction properties by mutagenesis of K12 and H31

Jenna-Claire Ellis, R. Paul Ross, Colin Hill

1.17 Summary

Nisin is a ribosomally synthesised Class I lantibiotic produced by *Lactococcus lactis* and is one of the most studied bacteriocins to date, largely due to its importance as a preservative within the food industry. Nisin is amenable to bioengineering due to its gene-encoded nature, and various studies have utilised this characteristic to create mutants with improved properties. In this study we started with an already created nisin variant termed nisin-AAA (nisin A with three Alanines (A) replacing the normal residues at positions 20, 21 and 22) and created a bank of variants with substitutions at positions 12 (Lysine 12, K12) and 31 (Histidine 31, H31). Substitutions were made both independently and in combination in an effort to determine the impact of each amino acid change on the ability of nisin to induce the P_{nisA} promoter (self-induction is an important property of nisin). We identified ten variants that retained the ability to induce P_{nisA} (K12K/H31H, K12V, K12Q, K12W, K12T, K12A, K12C, H31N, H31K and H31R). Further analysis suggested a role for amino acid charge on the peptides' induction capabilities. We also showed that six variants could not induce P_{nisA}, but were able to maintain induction once triggered by another peptide (K12M, K12I, K12L, K12R, K12S and K12Y). One mutant, K12V-AAA-H31V, which had previously been shown to be resistant to degradation by the proteases trypsin and chymotrypsin, also demonstrated loss of induction. With N, K and R representing known cut sites for trypsin or chymotrypsin and amino acid substitutions H, R, N and K at position 31 being the only substitutions capable of maintaining auto-induction, our results suggest that any substitution at position H31 of this variant will lead to either loss of auto-induction or digestion by chymotrypsin.

1.18 Introduction

Nisin A is a pentacyclic antimicrobial peptide that is ribosomally-synthesised by some *Lactococcus lactis* strains and which displays broad antimicrobial activity against Gram-positive bacteria[1]. This 34 amino acid bacteriocin is categorised as a Class I lantibiotic, due to the presence of one lanthionine and four methyllanthionine amino acid residues[1]. These unusual amino acids are a result of post-translational modifications which are enzymatically introduced when a covalent bridge is formed between a dehydrated serine (S) or threonine (T) residue and a cysteine (C) residue to become dehydroalanine (Dha) or dehydrobutyrine (Dhb), respectively[1].

The successful production of the mature nisin peptide is reliant on the inter-communication of several proteins and genes, and the mature peptide plays a role in its own induction through the specific activation of a number of these genes[1–4]. The transmembrane histidine protein kinase (HPK), NisK, is capable of detecting and binding the nisin peptide. This binding, involving the N-terminal ring structures of nisin, subsequently activates the intracellular response regulator, NisR, which in turn triggers the activation of the nisA promoter (P_{nisA}) on the nisin operon (Figure 2.1)[1–5]. In order to form the mature peptide, a precursor form of nisin with an N-terminal leader is first translated before being post-translationally modified. At this stage, selected S and T amino acids are dehydrated by NisB and lanthionine bridges are formed by NisC. The peptide is subsequently exported out of the cell by NisT and cleaved of its N-terminal leader by NisP. The resulting peptide is fully mature and can now interact with NisK resulting in an auto-induction loop (Figure 2.1)[1–4].

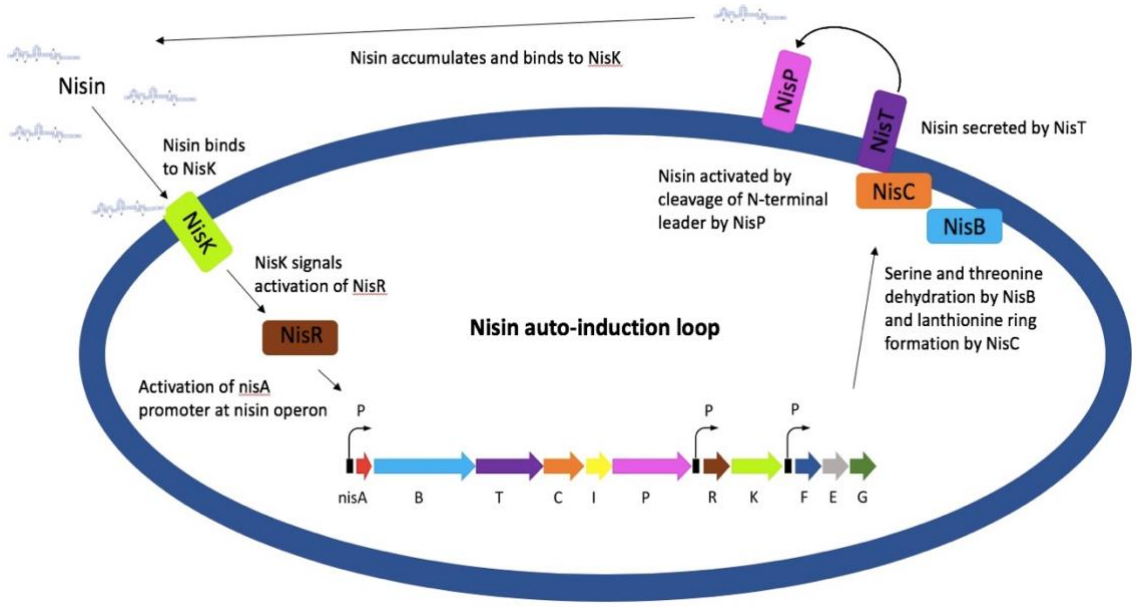


Figure 2.1 Nisin auto-induction loop schematic, adapted from Mierau et al., 2005[6].

For many years this nisin auto-induction mechanism, termed the Nisin-Controlled gene Expression (NICE) system, has been exploited for the expression of numerous genes. A gene of interest can be placed behind the inducible promoter P_{nisA} on a plasmid or the chromosome and, so long as NisRK are present, will be expressed when induced by the addition of sub-inhibitory amounts of nisin (0.1-5ng/ml)[6]. NZ9000 is the most commonly used host strain and has the signal transduction genes, *nisK* and *nisR* integrated into the *pepN* gene on the chromosome of MG1363 (nisin negative and plasmid free *L. lactis* subsp. *cremoris*)[3,6,7].

Its wide-ranging potential as a biological therapeutic has sparked interest in creating novel variants of nisin with higher activity, stability and solubility through bioengineering[8,9]. Due to their gene-encoded nature, many lantibiotics, including nisin, can be readily manipulated. In a previous study the amino acids asparagine (N),

methionine (M) and lysine (K) (referred to as the 'hinge' region between the N and C domains, located at residues 20-22) were changed to three alanine residues (AAA), resulting in a peptide with enhanced bioactivity against the bacterial strains *Lactococcus lactis* HP, *Streptococcus agalactiae* ATCC 13813, *Mycobacterium smegmatis* MC2155 and *Staphylococcus aureus* RF122[10]. A further study using this mutant (nisin-AAA) involved the alteration of the remaining cut sites for trypsin and chymotrypsin, thereby engineering the nisin-AAA mutant to become trypsin and chymotrypsin resistant. Several of these trypsin and chymotrypsin resistant variants lost their ability to induce P_{nisA}[11].

The nisin-AAA variant formed the basis of this study. A new round of genetic engineering was employed to generate 38 derivatives of nisin-AAA with all possible individual amino acid changes at positions K12 and H31 (Figure 2.2). In this study we examined the ability of these nisin variants to activate the auto-induction loop.

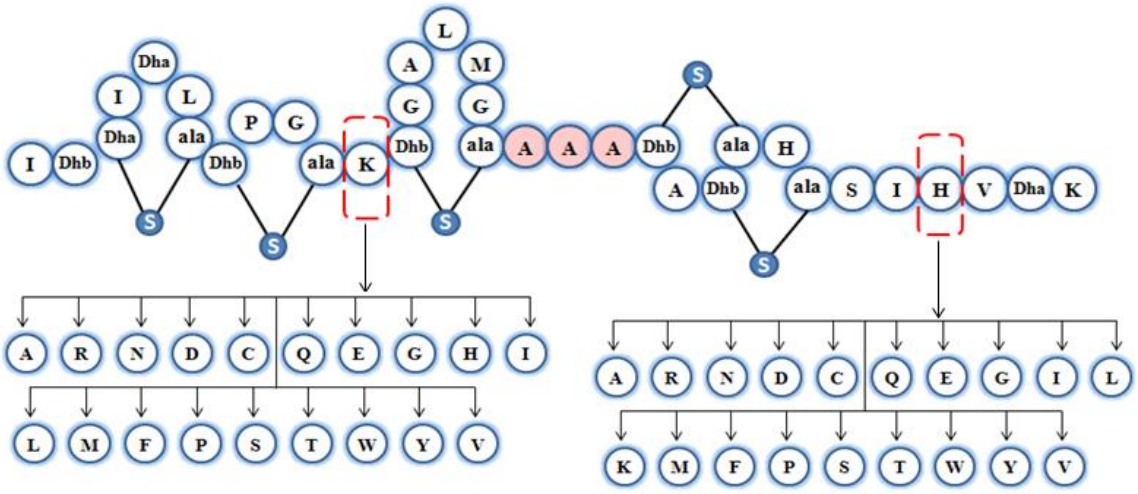


Figure 2.2 Nisin-AAA structure, consisting of the amino acid sequence of nisin A with the hinge region changed from NMK to AAA (highlighted in red), and the location of its five lanthionine rings and modified residues dehydroalanine and dehydrobutyrine. Positions K12 and H31 are boxed in red and show all amino acid changes made at these positions.

1.19 Experimental Procedures

1.19.1 Bacterial strains and growth conditions

The chemically competent *E. coli* Top 10 (Invitrogen) host strain was grown in Luria-Bertani (LB) broth or agar and incubated at 37°C. *Lactococcus lactis* strains (obtained from UCC culture collection) were grown in GM17 broth or agar (1.5% agar) (Oxoid) supplemented with 0.5% glucose and incubated at 30°C (Table 2.1). Tryptone yeast (TY) broth, used for peptide purification, was comprised of 3.125mg/ml Tryptone, 6.25mg/ml yeast extract, 62.5mg/ml manganese sulphate and 156.25mg/ml magnesium sulphate. In the incidences where chloramphenicol (CM) was required, a concentration of 10µg/ml was used for *E. coli* and 5µg/ml for *L. lactis*.

1.19.2 Site-saturation mutagenesis of the nisin-AAA K12X and H31X regions

Initially, the plasmid pDF05-AAA was extracted (using GeneJET plasmid mini Prep Kit, Thermo Scientific) from E. coli Top10 (Invitrogen), to ensure its methylation, and used as the template to make the separate targeted changes at positions lysine 12 (K12) and histidine 31 (H31) by means of randomized, site-saturated mutagenesis[10]. Phusion PCR was performed using primers NisK12degFOR and NisK12degREV, and NisH31degFOR and NisH31degREV (Table 2.2). PCR amplification was executed in a total of 50µl comprised of 2U/µl of Phusion High Fidelity DNA Polymerase (Novagen), ~50ng target DNA, 10X GC buffer, 10mM dNTPs and 200ng of the relevant oligonucleotides (Table 2.2). Cycling conditions were as follows: pre-heated at 98°C for 30secs, 98°C for 10secs, 67°C for 30secs and 72°C for 4.5mins for 34 cycles followed by a 72°C extension time for 10mins. The amplified product was treated with Dnp1

(Stratagene) along with buffer (UniV10X) at 37°C for 1hr to ensure the complete digest of parental DNA. A GeneJet PCR Purification Kit (ThermoFisher Scientific) was used to clean the PCR product and remove any remaining impurities. 2µl of mutated pCI372-NisAAA was introduced into Top10 chemically competent *E. coli* cells (Invitrogen) and plated on LB CM_{10} agar. To determine if the correct rate of mutation had been achieved, transformant colonies on LB CM_{10} agar were pooled (K12X and H31X transformants pooled separately) before isolating and sequencing recombinant plasmid DNA using primers pCI372FOR and pCI372REV (Table 2.2). Plasmid DNA was subsequently introduced by electroporation into electro-competent *L. lactis* MG1363. To identify specific amino acid changes the transformants were selected for on GM17 CM_5 agar and sent for sequencing (Source bioscience, Waterford, Ireland). Individual colonies were added to GM17 in 96-well microtiter plates and incubated over night at 30°C before adding 80% glycerol (Sigma Aldrich) and storing at -20°C. As many amino acid changes as feasible were made at positions K12 and H31 using this method, and the remaining variants were generated using site-directed mutagenesis (Table 2.2).

1.19.3 Site-directed mutagenesis of the nisin-AAA K12X and H31X regions

Remaining variants were generated through site-directed mutagenesis (K12P, K12F and K12M). Individual oligonucleotides were designed for each amino acid change and a 50µl Phusion PCR reaction was completed using the method as described above. The individual mutants were subsequently transformed into *E. coli* and *L. lactis* NZ9800 using the methods as described above.

1.19.4 Mass spectrometry

For Colony Mass Spectrometry (CMS) bacterial colonies were collected with sterile plastic loops and mixed with 50µl of 70% isopropanol adjusted to pH2 with HCl. The suspension was vortexed, the cells centrifuged at 14,000rpm for 2mins, and the supernatant removed for analysis. Mass Spectrometry in all cases was performed with an Axima CFR plus MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK). A 0.5µl aliquot of matrix solution (alpha-cyano-4-hydroxy cinnamic acid (CHCA), 10mg ml^{-1} in 50% acetonitrile-0.1% (v/v) trifluoroacetic acid) was placed onto the target and left for 1–2mins before being removed. The residual solution was then air-dried and the sample solution (re-suspended lyophilised powder or CMS supernatant) was positioned onto the pre-coated sample spot. Matrix solution (0.5µl) was added to the sample and allowed to air-dry. The sample was subsequently analysed in positive-ion reflectron mode.

1.19.5 Deferred antagonism assays

An agar-based deferred antagonism assay was used to analyse and compare the induction capabilities of the 38 nisin-AAA derivatives to the original nisin-AAA. Nisaplin (containing 2.5% nisin A) was used to induce the production of each nisin-AAA variant. Each bank of producers (full K12X bank and H31X bank) was stamped, using a replicator, onto GM17 agar plates both with and without 2.5µg/ml Nisaplin (0.0625µg/ml nisin A). Following incubation at 30°C for 18hrs the producer plates were UV treated for 30mins. GM17 agar (0.75% agar) was seeded with the indicator strain *L. lactis* HP and poured over the K12X and H31X producers followed by further incubation at 30°C for 18hrs (indicator strain growth conditions). The plates were examined for zones of clearance which indicate the susceptibility of the indicator strain to the nisin variant strain.

Zone size was measured using Vernier callipers and compared to the original nisin-AAA zone.

1.19.6 Agar well diffusion assays

4.6mm diameter wells were bored into appropriate agar that had previously been seeded with indicator strain at a 0.2% inoculum, and incubated at the appropriate temperature overnight. 20µl volumes of cell-free supernatant from overnight K12X and H31X nisin-AAA producing cultures were aliquoted into each well. Following 18hrs of incubation, at relevant temperatures, the zones produced were measured using Vernier callipers.

1.19.7 Induction signalling restoration

The ability of each new variant to maintain induction signalling was determined by growing K12X and H31X nisin-AAA producers with 2.5µg/ml Nisaplin (equivalent to 62.5ng/ml nisin) and incubating for 18hrs at 30°C using GM17 broth with 2.5µg/ml Nisaplin (without a variant) as a negative control. Following incubation, agar well diffusion assays were performed using *L. lactis* HP as an indicator strain as described previously. 10µl aliquots were also taken from overnight cultures and sub-cultured into fresh GM17 broth (without Nisaplin) and incubated for a further 18hrs at 30°C. Following this second incubation, a set of 10µl aliquots were sub-cultured once more into fresh GM17 broth. Well diffusion assays were performed on each set of sub-cultured overnights (method described previously). Zones produced following each incubation were measured using Vernier callipers.

1.19.8 Peptide purification

L. lactis NZ9700 (nisin A producer), or the mutant nisin strain of interest, was sub-cultured twice in GM17 broth at a 1% inoculum before use. Modified Tryptone Yeast (TY) broth was first made by supplementing TY broth with manganese sulphate (0.0625mg/ml) and magnesium sulphate (0.1562mg/ml) and subsequently passing the broth through a column (70cm length and 5cm internal diameter) packed to 1/3 its height with Amberlite XAD-16 beads (Sigma). Two litres of sterile modified TY broth were supplemented with glucose and β-glycerophosphate (0.01g/ml and 0.019g/ml respectively) before inoculation with the prepared culture at 0.5% and incubation at 30°C overnight. Following overnight incubation the culture was centrifuged for 15mins at 7000rpm. The supernatant was retained and the pelleted cells re-suspended in 300ml of 70% HPLC-grade 2-propanol containing 0.1% v/v trifluoroacetic acid (TFA) and stirred at room temperature for 3hrs. Cell debris was removed by a second centrifugation at 7000rpm for 15mins and the supernatant retained. The supernatants from the first and second centrifugation were combined and the volume reduced to approximately 300ml via rotary evaporation. The pH of the combined supernatants was adjusted to pH4 using sodium hydroxide (NaOH) and was subsequently passed through a 10g 60ml SPE C-18 column (Phenomenex) that had been pre-equilibrated with methanol and water. The column was washed with 120ml 30% ethanol, and the final solution containing the desired peptide was eluted in 60ml 70% 2-propanol containing 0.1% v/v TFA.

15ml aliquots of the final elution were concentrated through rotary-evaporation to approximately 2ml. 1.5ml samples were applied to a Phenomenex C12 Reverse-Phase (RP) HPLC column (Jupiter 4m proteo 90 Å, 250 X 10.0mm, 4μm) previously equilibrated with acetonitrile containing 0.1% v/v TFA. The solvent gradient was gradually increased from 25% to 60% at 45mins at a flow rate of 1.2ml/min. The resultant

fractions, which were deemed to contain peptide were pooled and excess solvent removed by rotary evaporation. Aliquots of the final solution were freeze dried at -50°C for approximately 48hrs.

1.19.9 Nisin induction using nisin A-GFP expression system

Growth and fluorescence assays using *L. lactis* NZ9000 pNZ8150-*gfp*+ containing the gene for green fluorescent protein (GFP) were completed using a 2% inoculum of *L. lactis* NZ9000 pNZ8150-*gfp*+ overnight in GM17 broth CM_{10}, followed by incubation for 3-4 hrs at 30°C. The OD_{600} was monitored to achieve an absorbance of ~ 0.2 – 0.3.

For supernatant assays both banks of variants (K12X and H31X), in addition to the original nisin A and nisin-AAA, were propagated overnight in GM17 CM_{10} and subsequently pelleted by centrifugation. The supernatant was collected and centrifuged a second time. 5µl aliquots were taken from each supernatant and added to 995µl fresh GM17 broth containing 20µl of the *L. lactis* NZ9800 pNZ8150-gfp+ (OD_{600} ~0.2-0.3 as previously outlined) and briefly vortexed. 200µl of each sample was aliquoted in triplicate into the wells of a 96 well plate and the fluorescence monitored over an 18hr period at 30°C using a SpectraMax MP3 spectrophotometer with an excitation/emission of 488/510nm. Green fluorescent protein was detected in terms of relative fluorescence units (RFU).

For peptide-based assays (K12V-AAA-H31V) 5µl of 5ng/ml, 10ng/ml and 100ng/ml concentrations of purified peptide were added to 995µl GM17 broth containing 20µl of the *L. Lactis* NZ9000 pNZ8150-*gfp*+ (OD_{600} ~0.2 - 0.3) and vortexed briefly. 200µl of each sample was aliquoted in triplicate into wells of a 96 well plate and the fluorescence monitored for 22hrs at 30°C as previously described.

1.20 Results

1.20.1 Generation, mass spectrometry and DNA sequencing analysis of a bank of nisin-AAA derivatives

Site-saturated and site-directed mutagenesis methods were used to generate a bank of nisin-AAA variants in which positions K12 and H31 were individually substituted with all 19 alternative natural amino acids (Tables 2.1, 2.2) (Figure 2.2). Site-saturation mutagenesis involved using oligonucleotides to replace the specific codons (at positions K12 and H31) with an NNK triplet potentially encoding for all 20 standard amino acids (Table 2). Variants that were not recovered using this method were subsequently made through site-directed mutagenesis using specific oligonucleotides, making up a complete "K12X" and "H31X" bank of producers (Table 2.2).

Mass spectrometry was performed on each variant to confirm each amino acid substitution (Tables 2.3, 2.4). Previous studies suggest that T and S, not normally present in mature nisin because of dehydration by NisB, retain the potential to be modified when introduced at positions 12 and 31, and can be dehydrated to form dehydroalanine (Dha) and dehydobutyrine (Dhb)[12]. In the presence of a C residue, intramolecular cross-links can occur covalently linking Dha or Dhb residues to C, thus forming lanthionine rings[4,13–15]. Analysis of K12S, K12T, H31S and H31T derivatives by colony mass spectrometry (CMS) confirmed these modifications occurred to some degree, with masses corresponding not only to unmodified S and T but also masses indicating the presence of Dha and Dhb respectively. In the case of derivative K12C the mass detected does not correspond to the calculated unmodified mass, thus indicating that the newly incorporated C has been modified. Also in line with results from previous studies, the variant K12D did not produce a corresponding mass[13]. DNA sequencing analysis confirmed that all 40

variants had been successfully introduced at the codons for the K12 and H31 positions with no other alterations to the nisin gene.

1.20.2 Analysis of bioactivity and self-induction of nisin-AAA bank

The bioactivity and auto-induction ability of each variant was assessed using deferred antagonism assays against the target indicator strain *L. lactis* HP. Nisin A (in the form of the commercially available Nisaplin at a concentration of 2.5µg/ml) was used to determine whether the production of each variant could be induced. In the absence of Nisaplin the auto-induction ability of each variant could be determined by the presence (retained auto-induction) or absence (lost auto-induction) of a zone of clearing (Figures 2.3, 2.4). It was observed that 11 of the 40 variants remain capable of self-induction. All variants demonstrated bioactivity once induced with Nisaplin, with the exception of K12D (Figure 2.4). In addition, each variant produced a larger zone of clearing when induced by Nisaplin as opposed to induction by its own peptide. We also noted that the variants K12V and K12C both produced larger zones when self-induced than original nisin-AAA.

1.20.3 Induction signalling restoration

Well diffusion assays were performed on Nisaplin-induced (2.5µg/ml) overnight (18hrs) cultures of each variant. Following the initial 18hrs incubation, 10µl was sub-cultured into fresh media (10ml) and grown for a further 18hrs followed by well diffusion assays. A second sub-culture lowered the Nisaplin concentration to 0.0025ng/ml. Well diffusion assays were subsequently performed on this second sub-culture. Our results showed that those variants originally capable of self-induction also continued to induce after the second passage (variants K12K, K12V, K12Q, K12W, K12T, K12M, K12A,

K12C, H31H, H31N, H31K and H31R). However, six variants at the K12X position, that had lost their ability to self-induce following mutagenesis, were able to retain production once initially induced by Nisin A, and maintained this through two subcultures; K12M, K12I, K12L, K12Y, K12R and K12S (Figure 2.5). Twelve variants retained their induction after the first passage, but subsequently lost this ability at the second passage (Figure 2.5).

1.20.4 Nisin-AAA derivative self-induction analysis using nisin GFP expression systems

A green fluorescent protein (GFP) expression reporter system (*L. Lactis* NZ9000 pNZ8150-*gfp*+ producer strain) was used to assess the induction abilities of each of the nisin-AAA variants (Figure 2.6)[16]. The highest level of fluorescence was determined for each variant (Figure 2.6). As expected, those variants able to self-induce also induced GFP expression. All K12X variants not capable of self-induction in previous assays, but able to retain activity after the second passage (0.0025ng/ml Nisaplin) (Figure 2.5 A), were also able to induce GFP production (Figure 2.6 A). Six variants that lost the ability to auto-induce following a second passage were still capable of inducing GFP production; K12H, H31Q, H31L, H31A, H31I and H31M. A lack of fluorescence signal was also observed in variants K12N, K12P, K12F, H31V, H31S and H31Y (Figure 2.6) which had lost their induction ability after the second passage (Nisaplin concentration of 0.0025ng/ml) in the previous assay (Figure 2.5). Two variants (H31T and H31P) that lost induction capabilities after the first passage (Nisaplin concentration of 0.0025μg/ml) (Figure 2.5 B), could induce GFP. It is also noteworthy that the positively charged amino acid changes were all able to induce GFP (Figure 2.6), even if they were not all able to

self-induce or retain self-induction after the second passage in the previous assay (Figure 2.5).

In one study variants were engineered with amino acid changes at both positions K12 and H31 to produce derivatives that were both trypsin and chymotrypsin resistant[11]. One of the most active variants, K12V-AAA-H31V, was evaluated in this study to determine its ability to maintain a self-induction loop. Simultaneously, the producing strain's growth, and corresponding peptide's ability to maintain a self-induction loop, were examined using absorbance growth curves and the GFP reporter system respectively, over 22hrs. It was observed that this variant was capable of inducing the production of GFP at a peptide concentration of 100ng/ml and only marginally induced GFP expression at a peptide concentration of 10ng/ml (Figure 2.7). When the peptide concentration was increased to 100ng/ml, nisin A had an extended lag time of >2hrs in the absorbance growth curve, which corresponds to the results observed in the GFP fluorescence curve (Figures 2.7 C, D), whereas the nisin K12V-AAA-H31V did not. When separately induced by 100ng/ml of each peptide, GFP expression reached approximately the same level of fluorescence (RLU) in each case albeit at different time points, and relative to the absorbance growth curve.

1.21 Discussion

Nisin has two domains, the N-terminal consisting of the A, B and C rings, and the C-terminal containing the D and E rings. The two domains are connected by a flexible hinge region, which in the nisin-AAA variant consists of three Alanines. Previous investigations involving nisin-AAA variants, show that selected combined amino acid changes at positions K12 and H31 give rise to trypsin and chymotrypsin resistance, whilst retaining biologically activity (one of the most active variants being nisin K12V-AAA-H31V)[11]. However, an unexpected side effect was the loss of auto-induction in the majority of the derivatives. The aim of this study was to determine what effects amino acid changes at K12 and H31 had on induction by analysing the expression of each nisin-AAA derivative in the presence and absence of Nisaplin, and also by monitoring the induction of a *gfp+* gene translationally fused to the nisin promoter.

Positions K12 and H31 of the nisin-AAA variant were separately changed to every possible alternative natural amino acid. Sequencing of each variant was performed to confirm the correct amino acid substitutions, before carrying out colony mass spectrometry to confirm the nature of each variant. The variant K12D failed to produce a mass or bioactivity, even when induced (Table 2.4 and Figure 2.5 A), which is consistent with previous studies[13,17]. In the case of T, S and C substitutions, post-translation modifications can occur to form Dha, Dhb and possibly lanthionine rings, which in the case of H31T, H31S, K12T and K12C variants was evident from the CMS results (Tables 3, 4)[4,13–15]. Further bioactivity assays were carried out on the K12C variant, however it was not more active against any indicator strains tested (with the exception of *L. lactis* HP), nor was it more soluble.

Determinations of the bioactivity of the variants against *L. Lactis* HP in the absence and presence of Nisaplin provided an indication of each variant's self-induction properties. When induced with 2.5µg/ml Nisaplin (containing 2.5% nisin A), all variants other than K12D demonstrated bioactivity as indicated by the presence of a zone of clearing against *L. lactis* HP[13,17,18]. When allowed to self-induce, both K12V and K12C were the only variants exhibiting zones of inhibition larger than the original nisin-AAA, possibly as a result of increased production, diffusion or specific activity. In the absence of Nisaplin only 10 nisin-AAA variants maintained the ability to self-induce (original nisin K12-AAA-H31, K12V, K12Q, K12W, K12T, K12A, K12C. H31N, H31K and H31R). Given that K12 is conserved across all known natural nisin variants, it implies that this is an important position for the peptide and its production[1,2]. Six substitutions at position K12 were capable of maintaining self-induction and bioactivity (K12V, K12Q, K12W, K12T, K12A and K12C). The introduction of the negatively charged amino acids D and E at position K12 resulted in the decrease or complete loss of production and self-induction (Figures 2.4 & 2.5). It is notable that in a previous study the nisin A K12E variant (with a charge of +4) was produced but did not exhibit activity, whereas in this study the nisin-AAA K12E variant (with a charge of +3) was not able to self-induce but was active once induced[13]. Changing the amino acid sequence of nisin A at the hinge region (from NMK to AAA) lowers the overall charge of the peptide, and thus could have an effect on the peptide's ability to induce and its bioactivity (i.e. the combined impact on production and activity). However, the nisin A K12E variant was not subjected to induction with Nisaplin, therefore the peptide may have been active but not produced at a high concentration. The introduction of the positively charged amino acids, K or R, at position H31 resulted in the retention of the self-induction property. It is relevant that sustained self-induction was exclusive only to the three natural positively charged amino

acids at position H31 (and N which is uncharged), suggesting that the positively charged amino acids at position H31 are important for maintaining self-induction. Previous studies investigating the effects of amino acid substitutions on the bioactivity and/or production of nisin have shown varying results[13,19]. By and large, substituting with a negatively charged amino acid has a detrimental effect on bioactivity/production, as in the cases of nisin Z hinge mutants N20E, M21E and K22E, nisin A mutants N20D and K22D, and nisin A mutant K12D[13,18–20]. However, one instance where negatively charged substitutions have had a beneficial effect with respect to specific activity relates to the derivatives S29D and S29E, which exhibited a two-fold decrease in MIC against lactococci[21]. Positively charged amino acid replacements have had a varied impact on the bioactivity/induction properties of nisin. For example, several studies have demonstrated the enhanced activity of variants with positively charged residues, most notably the nisin Z mutants N20K and M12K which displayed enhanced bioactivity against several Gram-negative bacteria[21,22]. Replacement of A 20 (N20) with R (N20R) resulted in reduced bioactivity against several targets, including staphylococci and streptococci[18]. Other evidence indicates that positively charged amino acids may not play a huge role in the induction activity of nisin, as shown in a previous study whereby the replacement of positively charged amino acids, K12 and H31, with an A residue demonstrated similar induction abilities than original nisin A[3]. However, our data substantiates the detrimental impact of introducing the negatively charged amino acids D and E at position K12 of the nisin-AAA variant, along with the potential importance of having a positively charged residue at position H31, with regards to induction. The importance of a positively charged amino acid at position K12, with regards to induction, in the nisin-AAA variant in comparison to the lack of importance in original nisin A may be due to the overall net charge of both peptides. Nisin-AAA is by nature a less positively charged peptide due to

replacing the positively charged K amino acid at position 22 with an A. The replacement of a second positively charged amino acid at position 12, decreasing the overall charge yet again, may be responsible for its loss of induction, in contrast to original nisin A retaining induction.

In addition to positions N20, M21 and K22, K12 and H31 have previously been shown to be trypsin and chymotrypsin cut sites, and altering these two positions results in resistance to cleavage by these enzymes[11]. From the deferred antagonism results, (Figure 2.4) several variants would potentially allow the peptide to retain its self-induction properties K12V, K12Q, K12W, K12T, K12A, K12C, H31N, H31K and H31R. Of these alternatives K, R and N are known to be targeted by trypsin, and the peptide bonds formed by aromatic residues Y, W and F (not naturally found in nisin) are cleaved by chymotrypsin[21,22]. In a previous study a variant with a Q substitution at position H31 was also shown to be susceptible to chymotrypsin[11]. Consequently, with just four possible advantageous substitutions remaining at position K12 (V, A, T and C), with regards to i) maintaining bioactivity, ii) acquiring trypsin and chymotrypsin resistance, and iii) sustaining self-induction, and none at position H31, it may be construed that a nisin-AAA variant acquiring each of these three attributes is not feasible. Added to this issue is the production of two variants, one modified (Dhb) and one unmodified, where T is present at position K12, both requiring separation and purification of each derivative for further analysis.

The deferred antagonism assay results indicate that all nisin-AAA variants, except K12D, could be produced following induction with wild type nisin. The ability of each nisin-AAA variant producer to sustain production through a self-induction loop was investigated by first “kick starting” the production of nisin with a minimal amount of Nisaplin and subsequently passaging the sample into fresh media, thus diluting the

concentration of Nisaplin present. Several variants failed to retain its auto-induction ability once the concentration of Nisaplin began to decrease (K12G, K12E, H31W, H31G, H31E, H31T, H31D, H31P, H21C and H31F), verifying that these variants lack the ability to induce in the absence of nisin peptide (Figure 2.5). Some variants exited the induction loop after a prolonged period. These variants were capable of signal production at a Nisaplin concentration of 0.0025μg/ml, however the signal appeared to be lost when the Nisaplin concentration fell to 0.0025ng/ml (K12N, K12H, K12P, K12F, H31V, H31Q, H31S, H21M, H31I, H31A, H21L and H31Y). The initial signalling but subsequent loss implies that these variants have some ability to signal induction, but either require a higher concentration or are unable to fully enable the induction loop. Along with variants that could initially self-induce without the aid of Nisaplin, there were six other variants that, once "kick started", could maintain the induction loop after Nisaplin concentrations were depleted (K12M, K12I, K12L, K12Y, K12R and K12S). All of these variants are K12 substitutions, suggesting that the H31 position is less amenable to change with regards to induction, with only four amino acid variations at H31 facilitating self-induction in comparison to thirteen at position K12 (Figure 2.5). Other than all variants being either neutral or positively charged, there appears to be no other obvious pattern (including size, polarity, position (surface or buried), aromatic, aliphatic, cyclic and acyclic) connecting these variants.

GFP expression was monitored during growth of the *L. lactis* NZ9000 pNZ8150-*gfp*+ producer strain, using each nisin-AAA K12 and H31 variant as an inducer which enabled us to examine the induction properties of the derivatives over a 24hr period. This analysis revealed that the same six variants capable of maintaining a self-induction loop once "kick started" (Figure 2.5) along with the original variants capable of self-induction (Figure 2.4), were able to induce a self-induction loop, detected by increased levels of

GFP (Figure 2.6). Interestingly, eight variants that could not maintain or initiate a self-induction loop in the previous assay (Figure 2.5), could "kick start" an auto-induction loop, again detected by increased levels of GFP (K12H, H31Q, H31L, H31A, H31I, H31M, H31T and H31P) (Figure 2.6).

Previous work has indicated that the variant K12V-AAA-H31V holds promise as a potential therapeutic, in that it is trypsin and chymotrypsin resistant whilst retaining much of its specific activity, and thus this variant became the focus of further investigation in this study[11]. It was observed that the K12V-AAA-H31V variant had to be used at a much higher concentration than that of original nisin A to induce GFP production (Figure 2.7), with nisin A initiating an induction response at 5ng/ml and K12V-AAA-H31V only producing a similar response at 100ng/ml[25]. However, a nisin A concentration as high as 100ng/ml appears to stress the cells, shown by the lag in growth, but does not in the case of the K12V-AAA-H31V mutant (Figure 2.7 C). The need to add more mutant peptide (~100ng/ml) to initiate induction, along with the lack of a lag in growth of the mutant peptide in comparison the original nisin A control, corroborates the finding that the mutant peptide is slightly less active (two-fold increase in MIC) than the original nisin A (Figures 2.7).

We show that substituting K for a V at position K12 has no detrimental effect on the K12V-AAA-H31V peptide's induction ability (Figures 2.4 A, 2.5 A and 2.6 A). However, substituting H for a V, or indeed any natural amino acid, at position H31 leads to a loss of the ability to initiate the induction loop (Figures 2.4 B, 2.5 B and 2.6 B). The amino acid substitutions H, R, N and K at position H31 are the only substitutions capable of maintaining the self-induction loop, and so we infer that nisin-AAA variants with a V at position K12 and either R, N or K at position H31 may be the best possible configuration for nisin-AAA to preserve both bioactivity and self-induction. However,

given the fact that N, K and R are known to be trypsin or chymotrypsin cut sites, we would predict that any substitution that is made at position H31 will result in either the loss of induction or result in the digestion by trypsin or chymotrypsin. Future work would require generating these variants (K12V-AAA-H31R, K12V-AAA-H31N and K12V-AAA-H31K) and testing each for self-induction, trypsin and chymotrypsin resistance, and retention of biological activity/bioactivity.

Plasmid/Strains	Characteristic	Growth Media	Reference source
pDF05 AAA	pCI372 with N20A/M21A/K22A substitutions in *nisA*	GM17	Healy *et al*., 2013
E. coli Top 10	Intermediate cloning host	LB	Invitrogen
L.lactis NZ9800	Cloning host	GM17	Gasson, 1983
L. lactis NZ9800 pDF05	Wild type nisin A producer	GM17	Field *et al*., 2008
L. lactis NZ9000 pNZ8150	*gfp*+ cloned into pNZ8150 and under control of the nisin promoter	GM17	UCC culture collection

Table 2.1 Plasmids and strains used in this study.

Oligonucleotide	Sequence
pCI372FOR	5’-cgggaagctagagtaagtag-3’
pCI372REV	5’-acctctcggttatgagttag-3’
NisK12degFOR	5’-pho- ccc ggt tgt **nnk** aca gga gct ctg atg ggt tgt gct gcg gct aca -3’
NisK12degREV	5’-tgt agc cgc agc aca acc cat cag agc tcc tgt **mnn** aca acc ggg -3’
NisH31degFOR	5’-pho- tgt agt att **nnk** gta agc aaa taa tct aga gtc gac ctg-3’
NisH31degREV	5’ ttt gct tac **mnn** aat act aca atg aca agt tgc tgt -3’
K12MFor	5'- ccc ggt tgt atg aca gga gct ctg atg ggt tgt gct gcg -3’
K12MRev	5'- agc tcc tgt cat aca acc ggg tgt aca tag cga aat act -3’
K12DFor	5'- ccc ggt tgt gac aca gga gct ctg atg ggt tgt gct gcg -3’
K12DRev	5'- agc tcc tgt gtc aca acc ggg tgt aca tag cga aat act -3’
H31FFor	5'- tgt agt att ttt gta agc aaa taa tct aga gtc gac ctg -3’
H31FRev	5'- ttt gct tac aaa aat act aca atg aca agt tgc tgt agc -3’
H31PFor	5'- tgt agt att ccc gta agc aaa taa tct aga gtc gac ctg -3’
H31PRev	5'- ttt gct tac ggg aat act aca atg aca agt tgc tgt agc -3’

Table 2.2 Pho -5’- Phosphate modification, **bold** – location for site-saturation mutagenesis, underlined – location for site-directed mutagenesis.

Variant	Calculated molecular mass	Actual molecular mass	Variant	Calculated molecular mass	Actual molecular mass
H31H	3193	3195	**H31C**	3159	3159
H31V	3155	3155	**H31S**	3143 3124*	- 3124*
H31N	3170	3170	**H31D**	3171	3175
H31K	3184	3184	**H31R**	3212	3212
H31Q	3184	3183	**H31Y**	3219	3219
H31W	3242	3241	**H31L**	3169	3169
H31G	3113	3112	**H31A**	3127	3127
H31T	3157 3138*	- 3138*	**H31I**	3169	3169
H31M	3187	3188	**H31P**	3153	3153
H31E	3185	3185	**H31F**	3203	3203

Table 2.3 Molecular masses of nisin-AAA variants at position H31. *Indicates dehydrated forms (hydrophobic modified residues), i.e. Dhb in the case of T (H31T) and Dha in the case of S (H31S).

Variant	Calculated molecular mass	Actual molecular mass	Variant	Calculated molecular mass	Actual molecular mass
K12H	3202	3202	**K12C**	3168	3193
K12V	3164	3164	**K12S**	3152 3133*	3151 -
K12N	3179	3179	**K12D**	3180	-
K12K	3193	3193	**K12R**	3221	3221
K12Q	3193	3193	**K12Y**	3228	3227
K12W	3251	3251	**K12L**	3178	3178
K12G	3122	3122	**K12A**	3136	3136
K12T	3165 3147*	3165 3147*	**K12I**	3178	3177
K12M	3196	3196	**K12P**	3162	3162
K12E	3194	3194	**K12F**	3212	3211

Table 2.4 Molecular masses of nisin-AAA variants at position K12. * Indicates dehydrated forms (hydrophobic modified residues), i.e. Dhb in the case of T (K12T) and Dha in the case of S (K12S).

Variant	Sequence
H31H	attacaagtatttcgctatgtacacccggttgtaaaacaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtatt**cac**gtaagcaaa
H31A	attacaagtatttcgctatgtacacccggttgtaaaacaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtatt**gcg**gtaagcaaa
H31C	attacaagtatttcgctatgtacacccggttgtaaaacaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtatt**tgt**gtaagcaaa
H31D	attacaagtatttcgctatgtacacccggttgtaaaacaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtatt**gat**gtaagcaaa
H31E	attacaagtatttcgctatgtacacccggttgtaaaacaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtatt**gag**gtaagcaaa
H31F	attacaagtatttcgctatgtacacccggttgtaaaacaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtatt**ttt**gtaagcaaa
H31G	attacaagtatttcgctatgtacacccggttgtaaaacaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtatt**ggg**gtaagcaaa
H31I	attacaagtatttcgctatgtacacccggttgtaaaacaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtatt**att**gtaagcaaa
H31K	attacaagtatttcgctatgtacacccggttgtaaaacaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtatt**aag**gtaagcaaa
H31L	attacaagtatttcgctatgtacacccggttgtaaaacaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtatt**ttg**gtaagcaaa

H31M	attacaagtatttcgctatgtacacccggttgtaaaacaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtatt**atg**gtaagcaaa
H31N	attacaagtatttcgctatgtacacccggttgtaaaacaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtatt**aat**gtaagcaaa
H31P	attacaagtatttcgctatgtacacccggttgtaaaacaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtatt**ccc**gtaagcaaa
H31Q	attacaagtatttcgctatgtacacccggttgtaaaacaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtatt**cag**gtaagcaaa
H31R	attacaagtatttcgctatgtacacccggttgtaaaacaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtatt**cgg**gtaagcaaa
H31S	attacaagtatttcgctatgtacacccggttgtaaaacaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtatt**tcg**gtaagcaaa
H31T	attacaagtatttcgctatgtacacccggttgtaaaacaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtatt**act**gtaagcaaa
H31V	attacaagtatttcgctatgtacacccggttgtaaaacaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtatt**gtg**gtaagcaaa
H31W	attacaagtatttcgctatgtacacccggttgtaaaacaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtatt**tgg**gtaagcaaa
H31Y	attacaagtatttcgctatgtacacccggttgtaaaacaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtatt**tat**gtaagcaaa

Table 2.5 Full sequence of nisin-AAA including mutations at position H31. Underlined – location of mutation site.

Variant	Sequence
K12K	attacaagtatttcgctatgtacacccggttgt**aaa**acaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtattcacgtaagcaaa
K12A	attacaagtatttcgctatgtacacccggttgt**gct**acaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtattcacgtaagcaaa
K12C	attacaagtatttcgctatgtacacccggttgt**tgt**acaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtattcacgtaagcaaa
K12D	attacaagtatttcgctatgtacacccggttgt**gat**acaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtattcacgtaagcaaa
K12E	attacaagtatttcgctatgtacacccggttgt**gag**acaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtattcacgtaagcaaa
K12F	attacaagtatttcgctatgtacacccggttgt**ttt**acaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtattcacgtaagcaaa
K12G	attacaagtatttcgctatgtacacccggttgt**ggg**acaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtattcacgtaagcaaa
K12H	attacaagtatttcgctatgtacacccggttgt**cat**acaggagctctgatgggttgtgctgcggctacagcaactngtcattgtagtattcacgtaagcaaa
K12I	attacaagtatttcgctatgtacacccggttgt**att**acaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtattcacgtaagcaaa

K12L	attacaagtatttcgctatgtacacccggttgt**ctg**acaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtattcacgtaagcaaa
K12M	attacaagtatttcgctatgtacacccggttgt**atg**acaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtattcacgtaagcaaa
K12N	attacaagtatttcgctatgtacacccggttgt**aat**acaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtattcacgtaagcaaa
K12P	attacaagtatttcgctatgtacacccggttgt**cct**acaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtattcacgtaagcaaa
K12Q	attacaagtatttcgctatgtacacccggttgt**cag**acaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtattcacgtaagcaaa
K12R	attacaagtatttcgctatgtacacccggttgt**cgt**acaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtattcacgtaagcaaa
K12S	attacaagtatttcgctatgtacacccggttgt**agt**acaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtattcacgtaagcaaa
K12T	attacaagtatttcgctatgtacacccggttgt**acg**acaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtattcacgtaagcaaa
K12W	attacaagtatttcgctatgtacacccggttgtt**tgg**acaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtattcacgtaagcaaa
K12Y	attacaagtatttcgctatgtacacccggttgtt**tat**acaggagctctgatgggttgtgctgcggctacagcaactngtcattgtagtattcacgtaagcaaa
K12V	attacaagtatttcgctatgtacacccggttgt**gtt**acaggagctctgatgggttgtgctgcggctacagcaacttgtcattgtagtattcacgtaagcaaa

Table 2.6 Full sequence of nisin-AAA including mutations at position K12. Underlined – location of mutation site.

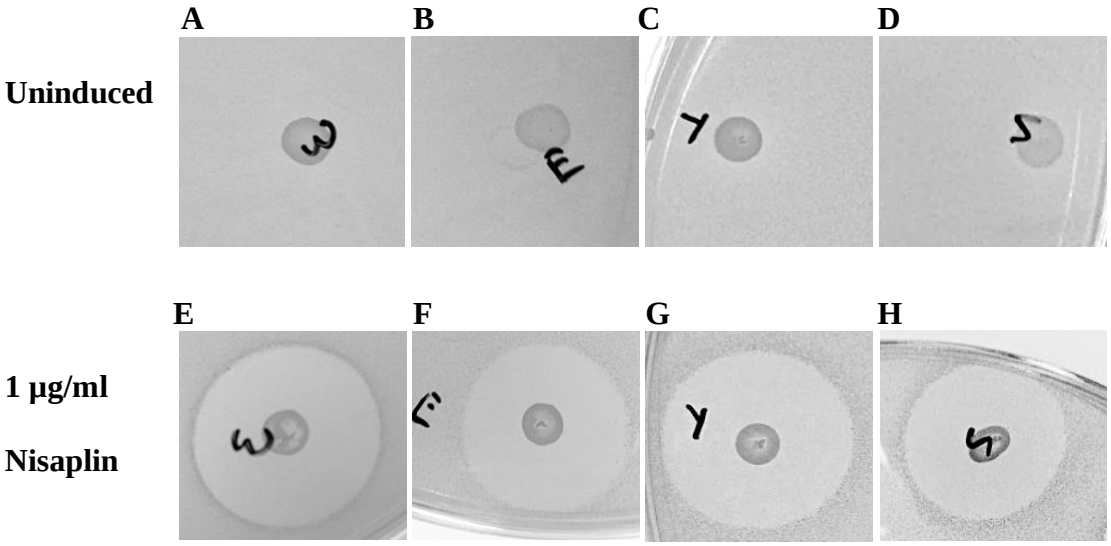
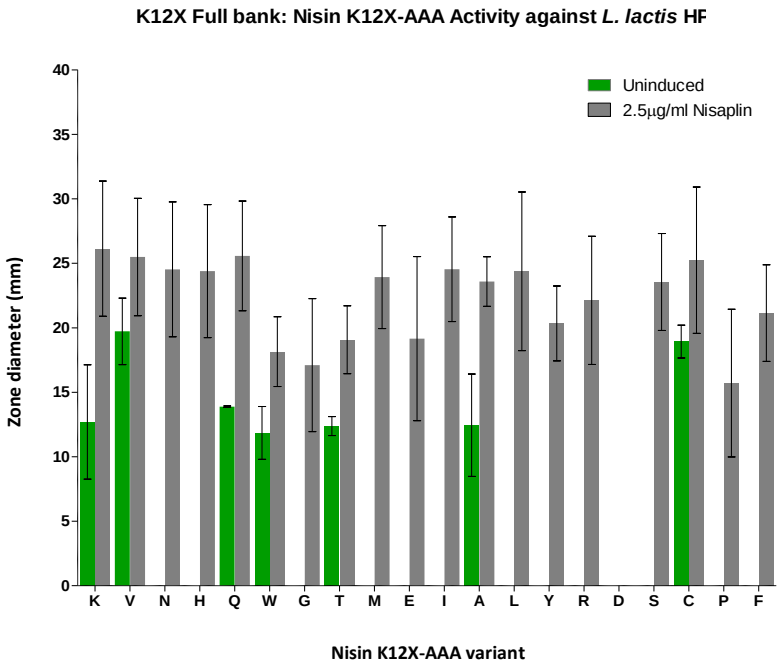


Figure 2.3 Deferred antagonism assay results showing zones of inhibition of nisin K12X-AAA and AAA-H31X variants against *L. lactis* HP. (A), (B), (C) and (D) depict uninduced variants K12W, K12E, H31Y and H31S respectively, plated on agar without Nisaplin. (E), (F), (G) and (H) depict induced variants K12W, K12E, H31Y and H31S respectively, plated on agar supplemented with 1µg/ml of Nisaplin (Danisco). Nisaplin contains a 2.5% concentration of nisin giving an overall nisin concentration of 0.025µg/ml.

A



B

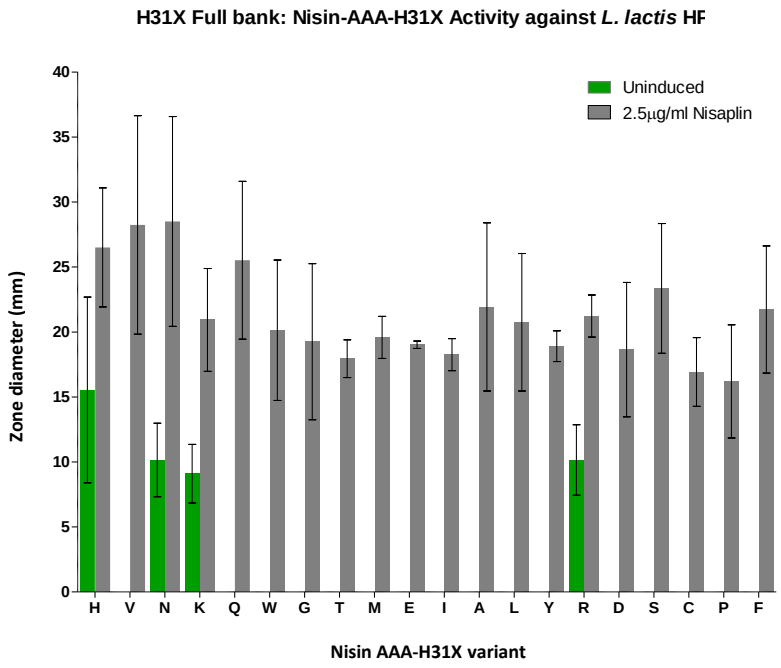
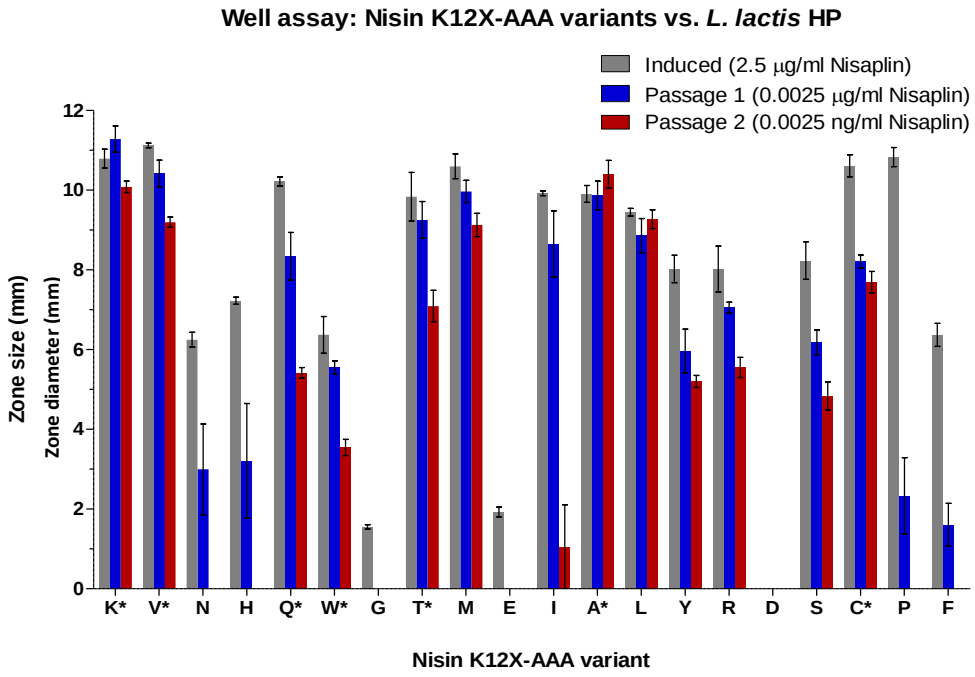


Figure 2.4 Deferred antagonism results of (A) nisin K12X-AAA and (B) nisin AAA-H31X variants supernatant against *L. lactis* HP. Results are expressed as the diameter (mm) of inhibitory zone minus the diameter of spot.

A



B

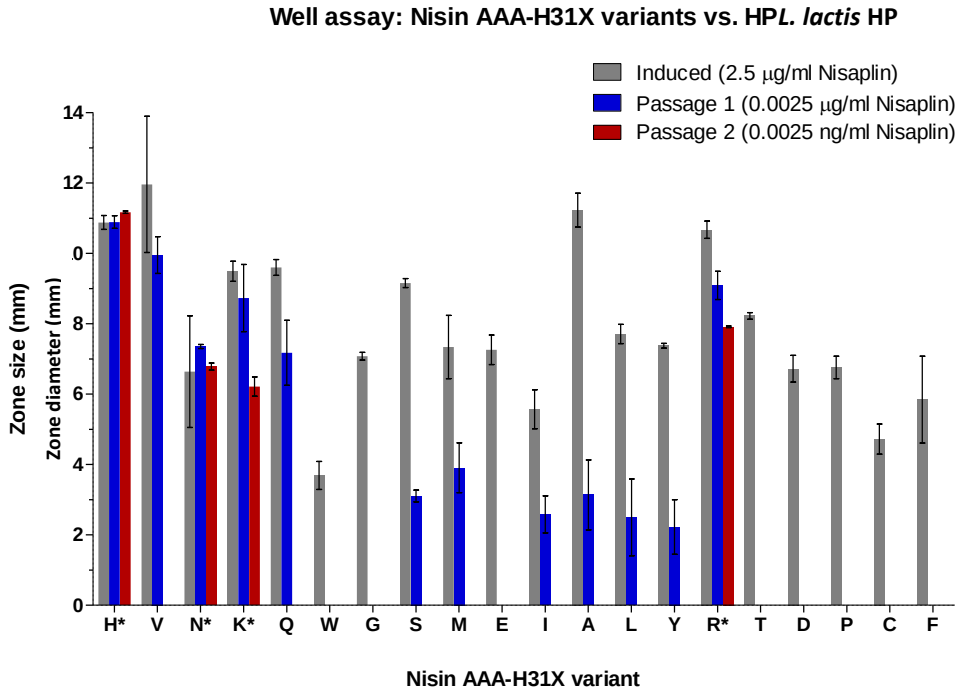
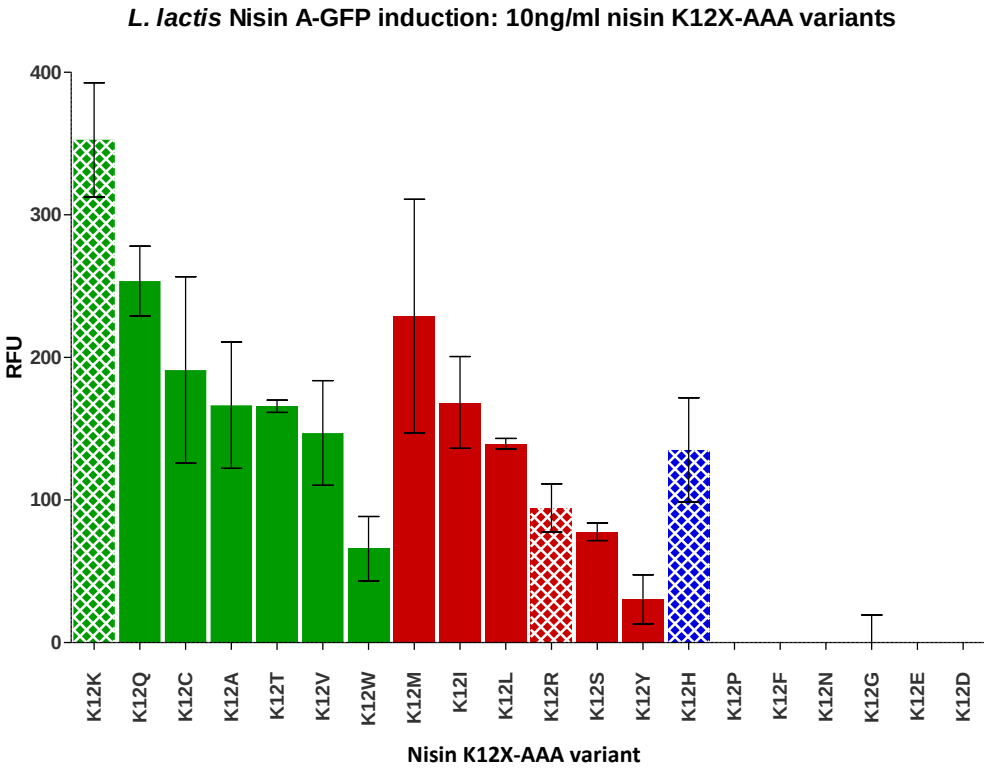


Figure 2.5 Well assay results of (A) K12X and (B) H31X variants against *L. Lactis* HP. Results are expressed as the diameter (mm) of inhibitory zone minus the diameter of well. *Indicates variants that initially self-induced without the addition of Nisaplin.

A


L. lactis Nisin A-GFP induction: 10ng/ml nisin K12X-AAA variants
RFU
400
300
200
100
0
K12K
K12Q
K12C
K12A
K12T
K12V
K12W
K12M
K12I
K12L
K12R
K12S
K12Y
K12H
K12P
K12F
K12N
K12G
K12E
K12D
Nisin K12X-AAA variant

B

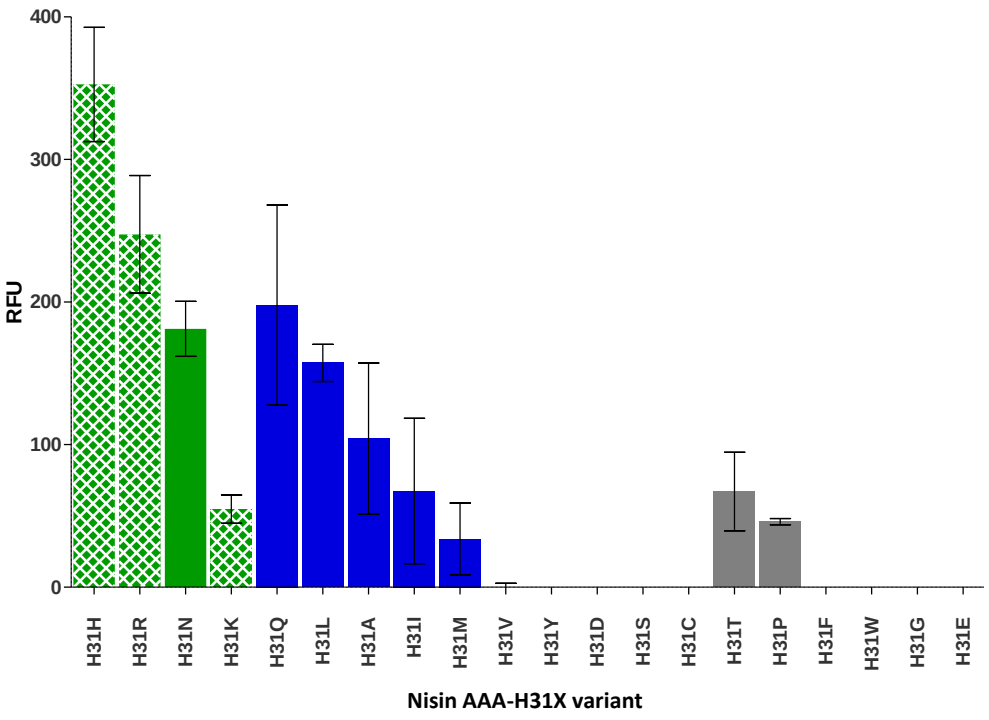

L. lactis Nisin A-GFP induction: 10ng/ml Nisin AAA-H31X variants
RFU
400
300
200
100
0
H31H
H31R
H31N
H31K
H31Q
H31L
H31A
H31I
H31M
H31V
H31Y
H31D
H31S
H31C
H31T
H31P
H31F
H31W
H31G
H31E
Nisin AAA-H31X variant

Figure 2.6 Results of nisin-A GFP induction using (A) K12X and (B) H31X variants. Green bars indicate naturally self-inducing variants; red bars indicate variants incapable of self-induction but capable of retaining induction when induced at 0.0025ng/ml Nisaplin (first passage); blue bars indicate variants incapable of self-induction but capable of retaining induction at 0.0025µg/ml Nisaplin (second passage); grey bars indicate variants incapable of self-induction but capable of retaining activity when induced with 2.5µg/ml Nisaplin; and patterned bars indicate positively charged amino acid changes.

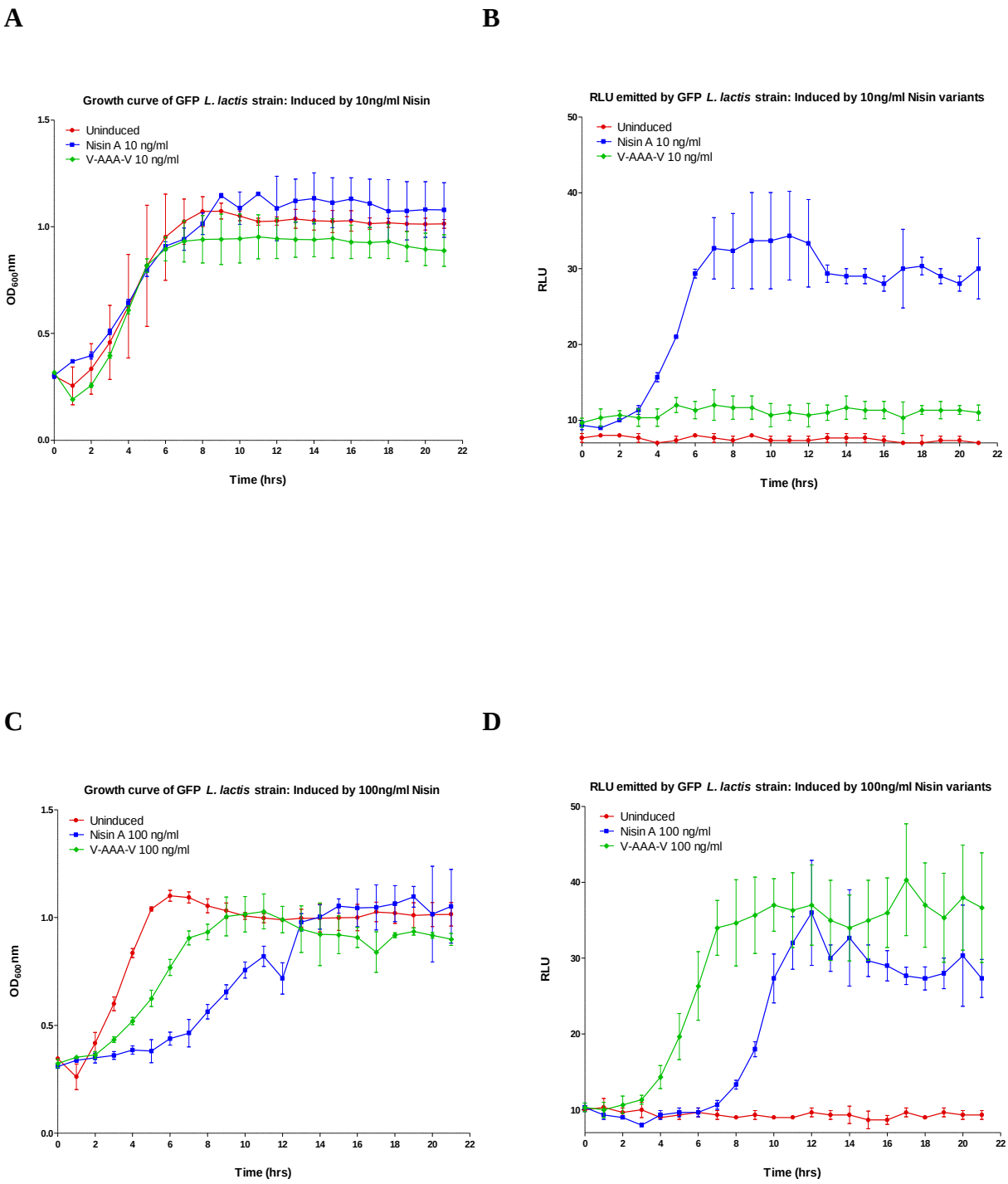


Figure 2.7 *In vitro* results of growth curves and nisin-A GFP expression, uninduced and induced with both nisin A and nisin K12V-AAA-H31V separately, using 10 ng/ml and 100 ng/ml concentrations. (A) and (C) represent absorbance growth curves whereas (B) and (D) represent RLU (Relative Light Units) curves.

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Chapter III

Conserving and restoring the use of antibiotics with the aid of bacteriocins.

Jenna-Claire Ellis, R. Paul Ross, Colin Hill

2.1 Summary

The threat of antibiotic resistance has become a global concern, with many clinically significant pathogens now showing increased resistance to multiple antibiotics. The search for alternative therapeutics has led to the analysis of possible solutions such as the use of bacteriocins. Bacteriocins typically have high potency, low toxicity and can be easily bioengineered making them ideal candidates. In this study 11 bacteriocins were tested for antimicrobial activity against five clinically significant bacteria. Nisin Z and lacticin 3147, which demonstrated the most promising results, were selected for combination studies using three different antibiotics (penicillin G, vancomycin and methicillin) *in vitro* and *ex vivo*. Results suggest that bacteriocins such as nisin Z and lacticin 3147 may aid in prolonging the use of certain antibiotics which are under threat of becoming ineffective due to antibiotic resistance, and can be used in combination with antibiotics that are no longer used clinically, such as methicillin, to revive the use of such antibiotics.

2.2 Introduction

Bacteriocins are bacterially produced, ribosomally synthesized, often post-translationally modified antimicrobial peptides, having either a broad or narrow spectrum of antimicrobial activity[1]. Their activity against numerous clinically significant pathogens, including various strains of methicillin resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis, Salmonella enterica* and *Clostridium difficile,* has heightened interest in their potential as therapeutics[1–7]. This is particularly relevant in an age of multi-drug resistant (MDR) bacteria combined with decreased efficacy of traditional antibiotics[2]. In recent years the growing issue of antibiotic resistance along with the disturbance of the human commensal microbiota through the administration of antibiotics has led researchers to investigate alternative therapies[8–10]. Other notable attributes of bacteriocins are their activity at relatively low concentrations, low toxicity and the possibility of *in situ* production by probiotics[2]. Studies examining the potiential use of bacteriocin producing probiotic bacteria, rather than purified bacteriocins, may also aid in making the use of bacteriocins more cost-effective.[11]

Nisin Z and Lacticin 3147 are both broad spectrum lantibiotics (class I bacteriocins) and are produced by *Lactococcus lactis*. Nisin has been used within the food industry as a food preservative since 1953 and has been approved by the EU, WHO and USFDA, making it an ideal candidate for further research as a potential therapeutic. Lacticin 3147 is comprised of two peptides, α and β, and demonstrates potent activity against a wide range of Gram-positive pathogens. Nisin and Lacticin 3147 have both exhibited *in vitro* activity against various clinically significant skin pathogens including MRSA, *Cutibacterium acnes* (previously referred to as *Propionibacterium acnes*), *Streptococcus pyogenes* and *Corynebacterium* species[12–16]. They have also previously

been shown to work synergistically with various other antibiotics against Gram-negative bacteria, such as polymyxin, amikacin and colistin[17–19].

Antibiotic resistance has become a global problem, so much so that governing bodies have started to take action. In 2015 the WHO (World Health Organisation) issued a "global action plan on antimicrobial resistance". After the "United Nations general assembly" in 2016 all member of states were required to implement their own national action plan for combating antibiotic-resistant bacteria by 2017. In 2015 the United States Federal Government put in place the "national action plan for combating antibiotic-resistant bacteria" and in 2017 the EU Commission began implementing the "EU one health action plan against AMR" which has been developed since 2011[20]. Both reports have commonalities pertaining to the importance of slowing the emergence of resistance bacteria, preventing the spread of resistant infections, accelerating basic and applied research, innovation and development for new antibiotics, other therapies and vaccines[21,22]. This study addresses these goals by investigating the potential for conserving the amount of antibiotic needed for the treatment of skin pathogens, developing new therapeutic strategies and restoring ones with now ineffective antimicrobial activity.

Cutibacterium acnes (*C. acnes*), previously classed as *Corynebacterium parvum* and more recently *Propionibacterium acnes,* is a Gram-positive, aerotolerant, commensal human skin bacterium capable of producing biofilms that contributes to the development of acne[23]. Acne affects 80% to 90% of the population at some stage in their life and is the primary reason for dermatologist visits[24,25]. Recent studies report the increasing emergence of *C. acnes* resistance to various antibiotics, including fluoroquinolones, macrolides, erythromycin, clindamycin, tetracycline and trimethoprim-sulfamethoxazole[26–28]. To date *C. acnes* remains susceptible to β-lactam antibiotics,

including penicillin. However, the possibility of acquiring resistance either through the alteration of the PBP (Penicillin binding protein) or gene transfer remains a cause for concern; strains have already been identified containing the β-lactam gene, which is not normally associated with Gram-positive bacteria[29]. Continual treatment with antibiotics such as penicillin, also increases the risk of other skin species developing resistance, for example the cutaneous staphylococcal flora of acne patients[30].

Corynebacterium xerosis (*C. xerosis*) is a commensal bacteria found on human skin and mucous membranes and rarely causes infections of any clinical relevance, however there have been reports of the bacteria contributing to strong underarm odours[31–33]. Previous studies have indicated that underarm odour was solely produced by aerobic coryneforms, the majority of which were identified as *C. xerosis*. In the same study 34 males were screened for underarm odour producing bacteria and 33 were positive for coryneforms, with population density strongly associated with the intensity of the odour[34]. *Enterococcus faecium* is another example of a bacterium that did not gain much attention until the mid to late 1990s when antibiotic resistant *E. faecium* nosocomial infections began to emerge with growing frequency, including aminoglycosides, ampicillin and vancomycin resistant *Enterococcus* (VRE) strains[35,36]. Enterococci can also be intrinsically resistant to a variety of antibiotics such as clindamycin, penicillin and cephalosporins, making their treatment more difficult.

While nosocomial and community acquired infections have always been cause for concern, the excessive and improper use of antibiotics has led to certain pathogens becoming increasingly difficult to manage[37]. MRSA is a Gram-positive coccus and has become one of the most worrying pathogens to date due to its highly mutable nature. In the 1940s *S. aureus* infections were originally treated with penicillin and later (1960s) penicillin-related antibiotics such as methicillin and oxacillin; however, new forms of the

pathogen resistant to β-lactam antibiotics (including penicillins, cephalosporins and carbapenams) began to emerge shortly thereafter[38–40]. Reports have shown more recent cases of resistance to other antibiotics such as erythromycin, fluoroquinolones, tetracycline and clydamicin[41–43]. Vancomycin is now the drug of choice to treat an MRSA infection, but a decreased susceptibility to this drug is also on the rise (known as vancomycin resistant *Staphylococcus aureus* (VRSA), with an Minimum Inhibitory Concentration (MIC) value of ≥32μg/ml rendering it completely resistant[43]. *Staphylococcus aureus* is ubiquitous in nature with approximately 20-30% of individuals displaying nasal passage colonisation persistently and 60% intermediately[37,44,45]. It causes a multitude of skin diseases ranging from superficial infections such as pimples, boils and impetigo to deeper skin infections such as abscesses and cellulitis. MRSA is also frequently associated with wound infections, including surgical site infections, and upon entering the bloodstream through cuts or lesions can lead to internal infections[46–48].

The threat of pandrug-resistance (resistance to all current drugs) is of growing concern for bacteria such as MRSA with 95% of strains not responding to first line antibiotics and increasing numbers of studies reporting resistance to alternatives such as vancomycin[49]. This study attempts to address this now global problem by using bacteriocins in combination with both currently used and now ineffective antibiotics in an effort to reduce toxicity and antibiotic resistance development, and to restore activity to antibiotics which have lost their efficacy.

2.3 Experimental procedures

2.3.1 Bacterial strains and growth conditions

All bacteriocin producing strains and pathogen strains are listed in tables 3.1 and 3.2 and were obtained from the UCC culture collection. *L. lactis* strains were grown in GM17 broth or agar (1.5% agar) (Oxoid) supplemented with 0.5% glucose and incubated at 30°C (Table 3.1). All *Streptococcus uberis* (*S. uberis*), *Streptococcus salivarius* (*S. salivarius*), *S. aureus*, *E. faecium* and *C. xerosis* strains were grown in BHI broth or agar (1.5% agar) and incubated at 37°C (Tables 3.1, 3.2). *C. xerosis* DPC 5629 was supplemented with tween80 0.5% v/v. *C. acnes* LMG 16711 was grown in Reinforced Clostridium Media (RCM) (Oxoid) and incubated at 37°C (Table 3.2).

Bacterial Strain	Bacteriocin	Media	Incubation temp.	Aerobic / anaerobic	Reference (s)
L. lactis MG1363 pMRCO1 pOMO2	Lacticin 3147	GM17	30°C	aerobic	([50])
L. lactis DPC5552	Lacticin 481	GM17	30°C	aerobic	([51])
L. lactis (NZ9700)	Nisin A	GM17	30°C	aerobic	([52])
L. lactis NZ9800 pCI372*nisF*	Nisin F	GM17	30°C	aerobic	([53])
L. lactis NZ9800 pCI372*nisQ*	Nisin Q	GM17	30°C	aerobic	([53])
L. lactis NZ9800 pCI372nisZ	Nisin Z	GM17	30°C	aerobic	([53])
S. uberis 42	Nisin U	BHI	37°C	aerobic	([54])
S. uberis D536	Nisin U2	BHI	37°C	aerobic	([53,54])
S. aureus C55	Staphylococcin C55	BHI	37°C	aerobic	([55])
S. aureus TY4	Staphylococcin TY4	BHI	37°C	aerobic	([56])
S. salivarius DPC6490	Salivaricin A5	BHI	37°C	anaerobic	([57])

Table 3.1 Bacteriocin producer strains and growth media used in this study. Brain Heart Infusion (BHI) (Oxoid) (1.5% agar) and GM17 (Oxoid) supplemented with 0.5% glucose (1.5% agar). All bacterial strains were obtained from the UCC culture collection.

Bacterial strain	Media	Incubation temp.	Aerobic/anaerobic
C. acnes LMG16711	RCM	37°C	anaerobic
C. xerosis DPC5629	BHI	37°C *	aerobic
E. faecium UCC1	BHI	37°C	aerobic
E. faecium UCC2	BHI	37°C	aerobic
MRSA ST291	BHI	37°C	aerobic

Table 3.2 Skin and gut relevant pathogens used in this study. Brain Heart infusion (BHI, Oxoid), Reinforced Clostridium Media (RCM, Oxoid). *Supplemented with tween80 0.5% v/v. All bacterial strains were obtained from the UCC culture collection.

2.3.2 Deferred antagonism

Following overnight incubation, the bacteriocin producer strain (Table 3.1) was spotted onto appropriate agar-containing media (Table 3.1) and incubated for 18hrs. Following incubation the producer strain splates were UV treated for 30mins. Indicator strains (*C. acnes* LMG 16711, *C. xerosis* DPC 5629, *E. faecium* UCC1, *E. faecium* UCC2 and *MRSA ST291*) were grown to stationary phase and 50µl was subsequently inoculated into 50ml of appropriate media (0.75% agar). This was then overlaid onto the bacteriocin producer strains plates. The overlaid plates were incubated overnight at the appropriate temperature and aerobic/anaerobic conditions for the indicator strains (Table 3.2). The results were represented as the diameter of the zone of inhibition minus the diameter of the bacteriocin producer spot and were measured using digital callipers (Figure 3.1).

2.3.3 Purification of Lacticin 3147

Following overnight incubation 10mls of *L. lactis* MG1363 (Lacticin 3147 pMRCO1 pOMO2) producing strain was added to 3 litres of Tryptone Yeast (TY) broth. 20X Glucose

(5%) and 20X β-glycerophosphate (5%) were also added and together incubated at 30°C for 16 hrs. The culture was subsequently centrifuged for 15 mins at 7000g. The pelleted cells were re-suspended in 300ml of 70% isopropanol (IPA) containing 0.1% v/v trifluoroacetic acid (TFA) and stirred at 4°C for 4 hrs before centrifugation at 7000 rpm for 15 mins. The volume of the supernatant was subsequently reduced by 70% using rotary evaporation (Buchi) (40˚C at 50mbar), in order to remove the IPA giving a final volume of ~ 60ml. The concentrated supernatant was subjected to solid-phase extraction (SPE) using a 10g/60ml Strata C-18E Giga-Tube (Phenomenex) post equilibration with 60ml methanol followed by 60ml H_2O. The column was subsequently washed with 120ml 30% ethanol and the peptide was eluted from the column with 60ml 70% IPA (pH 2).

For Reverse-Phase High Performance Liquid Chromatography (RP-HPLC), 10ml aliquots were concentrated through rotary-evaporation to ~2ml. Injection volumes of 1800µl were applied to a Phenomenex C_{12} Reverse-Phase (RP) HPLC column (Jupiter 4µ 90Å, 250 X 10.0mm, 4µm) previously equilibrated with 25% IPA containing 0.1% trifluoroacetic acid (TFA). The column was set up in a gradient of 30% IPA containing 0.1% TFA to 60% IPA containing 0.1% TFA in 4 to 40 mins at a flow rate of 1.2ml/min (Shimazdu, USA FMG Inc.). Fractions that generated a peak were pooled seperately and rotary-evaporated to remove approximately 40% of the IPA solvent before freeze-drying.

2.3.4 Minimum Inhibitory Concentration (MIC) assays

MIC assays were carried out in triplicate on Nisin Z and Lacticin 3147 using target strains *C. xerosis* DPC 5629, *C. acnes* LMG 16711, *E. faecium* UCC1, *E. faecium* UCC2 and MRSA ST291. 96 well microtitre plates (Sarstedt) were initially treated with bovine serum albumin (BSA) by adding 200µl of a 1% BSA and 5% PBS in sterile deionised water solution to each well, incubating at 37°C for 30 mins before decanting and washing with a 5% PBS in

water solution. Peptides and antibiotics were adjusted to a 5µM (16.67µg/ml nisin Z, 30.75µg/ml lacticin 3147, 1.78µg/ml penicillin G, and 7.245µg/ml vancomycin) starting concentration and a 2-fold serial dilution was performed on each peptide. The target strains were subcultured from an overnight (Table 3.2) and incubated until they reached an OD_{600} of 0.5nm. The strains were diluted in appropriate liquid media (Table 3.2) to a final concentration of 10^5 cfu/ml and 200µl was added to each well. The plate was incubated for 18hrs (Table 3.2) followed by MIC determination.

2.3.5 Growth Curve assays

Following overnight incubation, bacterial strains (Table 3.2) were transferred into appropriate liquid media (Table 3.2). Upon reaching an OD_{600} reading of 0.5nm, the cultures were supplemented with purified peptide (nisin Z/lacticin3147) and antibiotic (penicillin G/ vancomycin) (concentrations outlined in Figure 3.2), and 0.2ml was subsequently transferred to a 96 well microtitre plate (Sarstedt) in triplicate. Growth curves were measured spectrophotometrically, using a SpectraMax MP3 spectrophotometer and were plotted with incubation time as the abscissa and absorbance as the vertical axis.

2.3.6 Time-kill assays

Following overnight incubation, cultures were transferred into respective liquid media. Upon reaching an OD_{600} reading of 0.5nm, 20µl was transferred into 1 ml of appropriate broth (Table 3.2) containing purified peptide (nisin Z/ Lacticin 3147) alone and in combination with antibiotic (penicillin G/ vancomycin/ methicillin) (concentrations outlined in Figures 3.3, 3.4). The samples were incubated for 1 and 3 hrs, and/or 3, 6, 9, 12 and 24hrs at applicable temperatures (Table 3.2). Cell growth was measured by performing a 10-fold serial dilution of

cultures in one-quarter-strength Ringer solution and plating and enumerating viable cell counts on appropriate agar plates (Table 3.2)[58].

2.3.7 FIC/checkerboard assays

For FIC (Fractional Inhibitory Concentration) experiments, eight times the MIC of two antimicrobials (nisin Z and methicillin) were transferred separately into two 96 well microtitre plates upon which a serial dilution was then performed horizontally across each plate in broth (50µl). 50µl from plate 1 was added vertically to plate 2. The overnight culture (MRSA ST291), having been transferred into BHI media and grown until it reached an OD_{600} reading of 0.5nm, was diluted to a final concentration of 10^5 cfu/ml^{-1} and added to each well[17].

The FIC is characterised by the following equation:

FIC = FICX + FICY = (X/MICX) + (Y/MICY)

Where "X" is the minimum amount of antimicrobial X used in combination with another that achieved an inhibitory effect and the MICX is the MIC of the same antimicrobial that produces an inhibitory effect on its own. FIC index results are as follows: FIC $\leq$ 0.5 is synergy, 0.5 < FIC $\leq$ 0.75 is partial synergy, 0.75 < FIC $\leq$ 1.0 is additive, FIC > 1.0 is indifferent and FIC > 4 is antagonistic[17,59].

2.3.8 Ex-vivo *porcine skin model*

Approximately 2cm by 2cm squares of pig skin samples were cut with a scalpel and stored at -20°C. Samples were sterilised by soaking in 80% Propan-2-ol for 10mins before washing with deionised sterile water and subsequently exposing to UV light for 15mins. Samples were inoculated with OD_{600} 0.5nm (approximately 10^7cfu/ml) MRSA ST291 and incubated at 37°C for 1hr. 50µl of either 800µg/ml nisin and/or 32µg/ml methicillin was

pipetted onto the skin and spread over the surface using an inoculating loop before incubating at 37°C for 3hrs. Enumeration was carried out by homogenizing the skin in a stomacher (Masticator basic, IUL) with 10mls PBS for 15secs and performing plate counts on MRS agar incubated at 37°C for 18hrs.

2.4 Results

An initial screening using deferred antagonism agar diffusion assays was performed to determine if selected bacteriocins (Table 3.1) displayed antimicrobial activity against five pathogens (Figure 3.1). Each bacteriocin tested (Table 3.1) produced an antimicrobial effect against at least one of the five pathogens, thus suggesting possible bacteriocin production along with specific activity. Nisin Z and lacticin 3147 displayed zones of clearing greater in size, against four of the pathogens, than the majority of bacteriocins being considered, and were therefore selected for further investigation. It should also be noted that no bacteriocin producer produced a zone of clearing against the strain MRSA ST291.

In order to assess specific activity, high performance liquid chromatography (HPLC) and freeze-drying was carried to generate purified peptide before using the peptide in broth based 96 well microtitre plate assays to determine MICs against the same five indicator strains (Table 3.2). The MIC was visually determined as the lowest concentration of peptide that resulted in the absence of growth of the target strain after 18hrs of incubation at 37°C. It was apparent that for *C. acnes* LMG 16711, both antibiotics, penicillin G and vancomycin, had a greater antimicrobial effect than either bacteriocin (nisin Z or lacticin 3147) with nisin Z being the more effective of the two having an MIC difference of 0.625μg/ml when compared to vancomycin (Table 3.3). In the case of both *E. faecium* strains both nisin Z and lacticin 3147 produced a greater antimicrobial effect than either antibiotic with lacticin 3147 giving the lowest MIC values of 0.12μg/ml and 0.48μg/ml (Table 3.3). Lacticin 3147 again produced the lowest MIC value against *C. xerosis* DPC 5629 and had a lower MIC value than both nisin Z and penicillin G when tested against MRSA ST291. However, vancomycin remains the most effective antimicrobial for MRSA ST291 that was tested in this study (Table 3.3).

From the MIC results, lacticin 3147 was selected for further analysis by combination growth curve and time-kill assays with penicillin G and vancomycin against the strains *C. acnes* LMG 16711, *E. faecium* UCC1, *E. faecium* UCC2, *C. xerosis* DPC 5629and MRSA ST291 (Figures 3.2, 3.3). In each instance, when the peptide and antibiotic were used singly at sub-lethal concentrations, there was an increase of lag time and/or lack of full growth potential. However, when lacticin 3147 and an antibiotic (penicillin G or vancomycin) were used in combination, compared to either compound used alone, growth appeared to be completely inhibited. This was true for all five strains tested. In order to examine the antimicrobial effects of lacticin 3147 in combination with either penicillin G or vancomycin over a defined time period, each test strain (Table 3.2) was exposed to different concentrations of lacticin 3147 and antibiotic (penicillin G or vancomycin) in an appropriate broth for a period of 1 or 3hrs at 37°C (Figure 3.3). In the case of all five test strains, when lacticin 3147 was used in combination with antibiotic, cell numbers decreased to a greater extent to when they were used alone. In the case of both *C. acnes* LMG 16711 and *C. xerosis* DPC5629 after 3hrs of treatment, cell numbers remained static or slightly decreased (in comparison to untreated control) when treated with 0.176μg/ml penicillin G (*C. acnes* LMG 16711) or 0.905μg/ml vancomycin (*C. xerosis* DPC 5629) and decreased by 2-log when treated with 7.5μg/ml (*C. acnes* LMG 16711) or 1.2μg/ml (*C. xerosis* DPC 5629*)* Lacticin 3147. However cell numbers decreased by >5-log when treated in combination with either penicillin G (*C. acnes* LMG 16711) or vancomycin (*C. xerosis* DPC 5629) (Figures 3.3 A, 3.3 C). After 3hrs *E. faecium* UCC2 cell counts also decreased significantly (>8-log) when lacticin 3147 and penicillin G were used in combination, in comparison to only decreasing by a maximum of 5-log when treated with either alone. For *E. faecium* UCC1 at a 3hr time point there was less of a decrease in cfu/ml when lacticin 3147 and vancomycin were used in combination in comparison to when used alone, which is consistent with results from growth curves (Figure 3.2). At a time point of 3hrs MRSA ST291

displayed a decrease of 2-log when treated with vancomycin alone, and 5-log when treated with lacticin 3147 alone, in comparison to a 6-log when in combination.

In a further attempt to demonstrate the use of bacteriocins for the enhancement of antibiotic efficacy, FIC assays were implemented in order to determine whether a bacteriocin can work synergistically with an antibiotic against a bacteria that is known to be resistant to the same antibiotic. In this case nisin Z was chosen because it has already been approved by the FDA, making it a potentially more easily and rapidly implemented therapeutic. The MIC of >64μg/ml for methicillin confirms that the *S. aureus* strain used is indeed an MRSA. The FIC assays confirmed that a combination of vancomycin and nisin Z had only an additive effect on MRSA ST291 (FIC 1.01), while a combination of methicillin and nisin Z gave a more promising result of having a partially synergistic effect (FIC 0.75) (Table 3.3).

Further time-kill assays were carried out to establish the effect of nisin Z and methicillin alone or in combination on MRSA ST291 over 3, 6, 9, 12 and 24hr periods (Figure 3.4 A). It was ascertained that over a 24hr period nisin Z at a concentration of 1μg/ml and methicillin at a concentration of 32μg/ml did not have significant inhibitory effects on the growth of MRSA ST291, but when used in combination gave a 5-log cfu/ml reduction. At the 3hr time point nisin Z and methicillin alone had a maximum of a 2-log difference. However, over the other time points (6, 9, 12 and 24hrs) it can be seen that both nisin Z and methicillin alone could not maintain this effect and there is little to no difference in cfu/ml at the 24hr time point. This is in contrast to when nisin Z and methicillin were used in combination in which case at the 12hr time point the difference in cfu/ml is still 5-log. By the 24hr time point colony counts of nisin and methicillin, in combination, treated cells were equal to that of untreated cells, however, colonies appear to be smaller indicating that the cells are stressed (Figure 3.4 B).

An additional assay was carried out in order to determine if the benefits of combining nisin Z and methicillin extended to when applied to an ex vivo porcine skin model. The results

showed that when nisin Z and methicillin were applied to pig skin that had been infected with MRSA ST291, at a concentration of 1μg/ml and 32μg/ml respectively, that there was a greater antimicrobial effect when used in combination than either displayed alone (Figure 3.5).

2.5 Discussion

Considering the effects of antibiotic over-exposure in the environment, in agriculture and in healthcare settings on antibiotic resistance, alternative therapeutic options need to be explored. The potential of using bacteriocins as biological therapeutics is attracting an increasing amount of attention due to their low toxicity and highly potent and stable nature. Previous studies involving combinations of bacteriocins and other antimicrobials have shown promise; such as the bacteriocin duracin 61A demonstrating synergistic activity against MRSA (*S. aureus* ATCC 700699) when used in combination with vancomycin, and the synergistic effect between nisin and penicillin when used in combination against multiple *E. faecalis* strains[60,61]. In this study we further examined the use of bacteriocins in combination with different antibiotics for the treatment of various pathogens. An initial screening for antimicrobial activity using a bank of bacteriocins against five different strains of bacteria highlighted the potential of two bacteriocins, nisin Z and lacticin 3147, for antibiotic combination studies. Nisin Z demonstrated the highest level of activity against *C. acnes* LMG 16711 and otherwise comparable activity to the two other bacteriocins with the highest level of activity against *E. faecium* UCC2 (nisin F and lacticin 3147). Lacticin 3147 demonstrated the highest level of activity against *E. faecium* UCC1 and *C. xerosis* DPC 5629 and exhibited comparable activity to the two other bacteriocins with the highest level of activity against *E. faecium* UCC2 (nisin F and nisin Z). For these reasons nisin Z and lacticin 3147 were both chosen for further combination studies with a number of antibiotics. It is also noteworthy that in this initial screen no bacteriocin demonstrated activity against MRSA ST291.

MIC results demonstrated that all strains used in this study remain sensitive to both penicillin G and vancomycin, with the exception of MRSA ST291 which exhibited resistance to penicillin G. In the case of *C. acnes* LMG 16711 a higher concentration of bacteriocin was

required to produce an antimicrobial effect than antibiotic. However, for the other four strains tested some bacteriocins proved to be more effective than the antibiotics tested, as was in the case of both nisin Z and lacticin 3147 against *E. faecium* UCC1 and *E. faecium* UCC2 and lacticin 3147 against *C. xerosis* DPC 5629.

In this study the amount of antibiotic, such as vancomycin or penicillin (associated with toxicity in patients and a growing concern of resistance), required to kill the pathogens was reduced by the addition of bacteriocins such as nisin Z and lacticin 3147, which have exhibited no adverse effects in animals to date[62]. Both nisin and lacticin 3147 produce antimicrobial activity by interacting with lipid II, forming pores in the cell membrane and inhibiting cell wall synthesis. These pores are thought to facilitate intracellular access to antibiotics allowing them to act more effectively and thus producing a synergistic effect. The increased efficiency of either penicillin G or vancomycin when used in combination with lacticin 3147 was observed using growth curves (Figure 3.2). The antimicrobial activity of penicillin G and vancomycin against each bacterial strain tested was significantly strengthened in the presence of lacticin 3147. Over the 48hr period, or 100hr period in the case of *C. acnes* LMG 16711, the level of bacterial growth when treated with either lacticin 3147 or antibiotic alone was substantial when compared to the lack of growth when treated with a combination. In the case of *C. acnes* LMG 16711 the concentration of penicillin G could be reduced from 0.2μg/ml to 0.015μg/ml when used in combination with 1.5μg/ml lacticin 3147. For both *E. faecium* strains penicillin G could be reduced from 3.56μg/ml to 0.22μg/ml when used in combination with 1.8μg/ml lacticin 3147 (*E. faecium* UCC1) or 2.4μg/ml (*E. faecium* UCC2). The concentration of vancomycin could be reduced from 0.91μg/ml to 0.45μg/ml when used in combination with 1.2μg/ml lacticin 3147 for the treatment of *C. xerosis* DPC 5629, and from 1.81μg/ml to 0.91μg/ml when used in combination with 38.5μg/ml lacticin 3147 for the treatment of MRSA ST291. Penicillin acts by binding to a penicillin-binding protein (PBP), DD-transpeptidase, preventing cell wall

synthesis, whereas vancomycin acts by binding to D-alanyl-D-alanine containing peptides and preventing their incorporation into the peptidoglycan matrix of the cell wall, thus preventing cell wall biosynthesis[63,64]. The bacteria, after encountering multiple approaches of attack on its cell wall from both lacticin 3147 and antibiotic, cannot recover as easily as with one approach and thus a higher rate of death occurs. This can be seen in both the growth curves and time-kill assays (Figures 3.2, 3.3), where it is evident that the same concentration of either lacticin 3147 or antibiotic (penicillin G or vancomycin) have displayed greater antimicrobial activity, to a varying degree, against each indicator strain when used in combination than either used alone. Combining antimicrobials with different antimicrobial mechanisms rather than antimicrobials with the same or similar modes of action in general yield better antimicrobial results along with reducing the prospect of resistance development[61,65,66]. Previous studies have shown that combining vancomycin, which by inhibiting cell wall synthesis decreases access to lipid II, with a bacteriocin that interacts with lipid II, such as lacticin 3147 or nisin, can decrease the possibility of synergistic antimicrobial activity[61]. Therefore combining lacticin 3147 or nisin Z with an antibiotic such as penicillin, which attacks by a different pathway, may be a better approach to achieve synergy.

Along with reducing the amount of antibiotic required, this study focused on the possibility of using antibiotics, to which bacteria have already established resistance, in combination with bacteriocins in an effort to reclaim the use of such antibiotics. Due to MRSA's clinical significance, methicillin was used as the subject for investigation in combination with the bacteriocin nisin Z for the treatment of MRSA. Nisin and methicillin attack the cell wall synthesis pathway at different points, thus potentially allowing them to generate a greater disruption within the cell wall when used together. FIC results confirm partial synergy between nisin Z and methicillin against MRSA ST291 (Table 3.3). FIC results also show a lack of synergy between nisin Z and vancomycin, possibly due to vancomycin

decreasing the accessibility of lipid II to nisin Z (Table 3.3). Time-kill assays were used to further analyse nisin Z and methicillin's activity against MRSA ST291 over a 24hr period, with a decrease in growth when used in combination compared to either alone. MRSA ST291, when treated with either nisin Z or methicillin, reached the same level of growth as the untreated control between 9 and 12hrs, whereas it did not reach the same level of growth until ~24hrs when treated with both (Figure 3.4 A). Despite the fact that the cfu/ml of the combination-treated cells was equivalent to that of cells treated with either alone the colonies were much smaller, indicating they were stressed (Figure 3.4 B). Interestingly the cfu/ml of the cells treated alone continually rose over the 24hr period, however the cfu/ml of the combination treated cells declined until the 9hr point at which it then began the rise, indicating that the combination of nisin Z and methicillin worked optimally up until the 9hr time point.

The enhanced antimicrobial activity of nisin Z and methicillin when used in combination was further analysed for its potential clinical use by means of an *ex vivo* porcine skin model. When applied to MRSA ST291 infected porcine skin the combination of antimicrobials (nisin Z and methicillin) had a greater effect than the sum of antimicrobial activity of either alone (Figure 3.5). However, the antimicrobial effect of both nisin Z and methicillin appear to be reduced when applied to the skin model, with the difference in cfu/ml being much less than that achieved *in vitro*. Nevertheless, a greater antimicrobial effect can still be seen, suggesting that the model may need to be optimised and further analysis carried out.

The exact mechanism by which nisin Z and methicillin produce a synergistic effect against MRSA ST291 is still unclear, as MRSAs resistance to methicillin is reliant upon its ability to produce the enzyme β-lactamase, which results in the digestion of methicillin rendering it inactive against MRSA. Further studies need to be done to determine if, in the presence of both methicillin and nisin Z, the production of β-lactamase is down regulated,

possibly as a result of cellular stress, thus allowing for methicillin to again have antimicrobial activity against MRSA. Similar studies should be done for when using lacticin 3147 in combination with penicillin G against β-lactamase producing pathogens.

With antibiotic resistance becoming of greater concern the need for alternative therapies is becoming more prevalent. These *in vitro* and *ex vivo* results suggest that bacteriocins such as nisin Z and lacticin 3147 may i) aid in prolonging the use of certain antibiotics which are under threat of becoming ineffective due to antibiotic resistance and ii) be used in combination with currently ineffective antibiotics, like methicillin, to revive the use of such antibiotics.

Organism	MIC (µg/ml)				FIC	
	Nisin Z	Lacticin 3147	Penicillin G	Vancomycin	Nisin Z / methicillin	Nisin Z / vancomycin
C. acnes LMG 16711	1.88	2.50	0.06 (S)	1.25 (S)	-	-
E. faecium UCC1	0.26	0.12	0.89 (S)	0.63 (S)	-	-
C. xerosis DPC 5629	8.33	0.24	0.63 (R)	0.45 (S)	-	-
E. faecium UCC2	0.52	0.48	0.89 (S)	0.63 (S)	-	-
MRSA ST291	4.17	3.85	20.00 (R)	0.91 (S)	0.75[a]	1.01[b]

Table 3.3 MIC - Minimum Inhibitory Concentration results of nisin Z, Lacticin 3147, Penicillin G and Vancomycin against Gram-positive strains *Cutibacterium acnes* LMG 16711, *Enterococcus faecium* UCC1, *Corynebacterium xerosis* DPC 5629, *Enterococcus faecium* UCC2 and MRSA ST291. Resistant (R) and Sensitive (S) values are taken from EUCAST clinical breakpoint 2019 report[67]. FIC – Fractional Inhibitory Concentration results indicate [a]partial synergy and [b]additive.

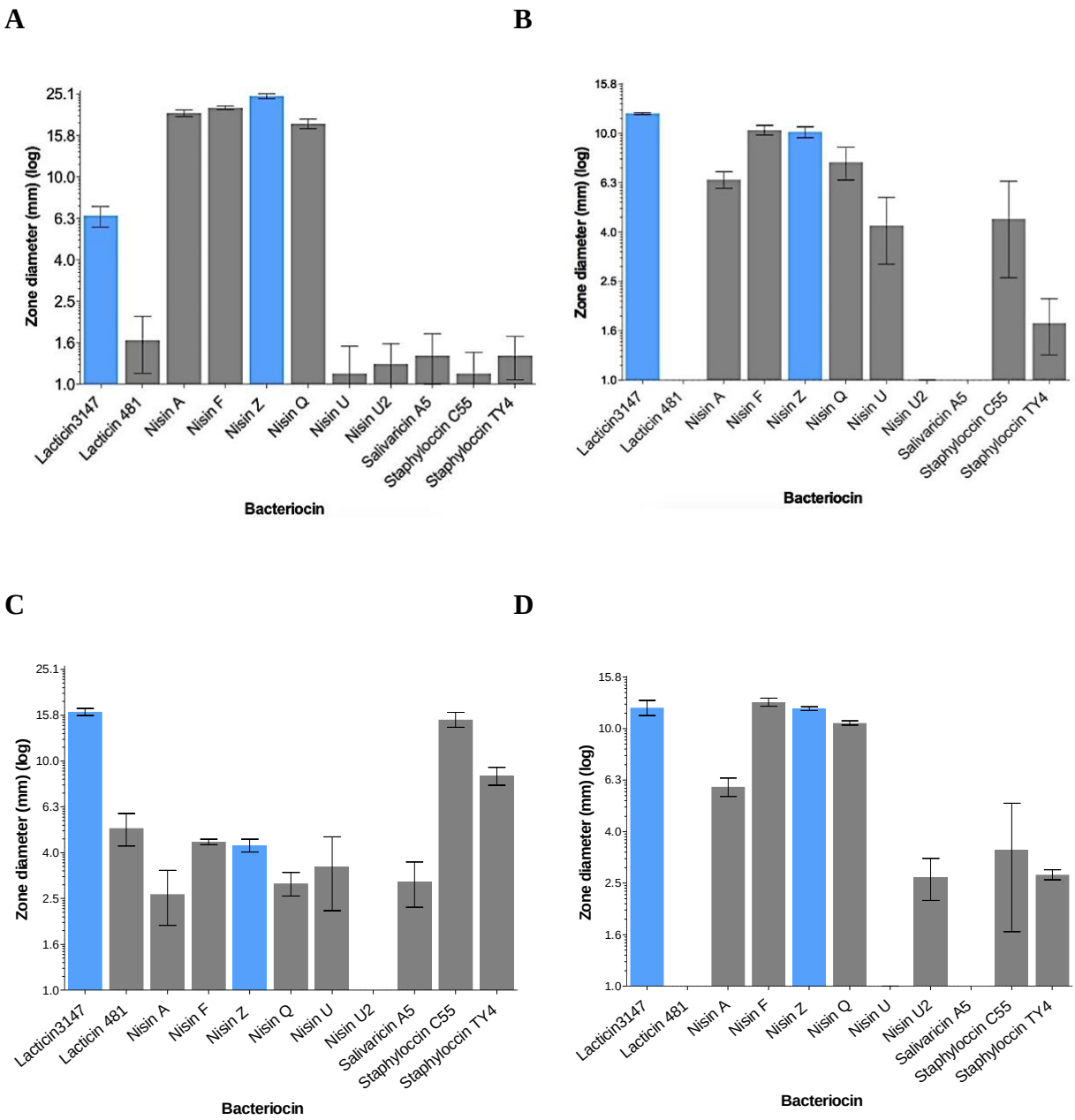
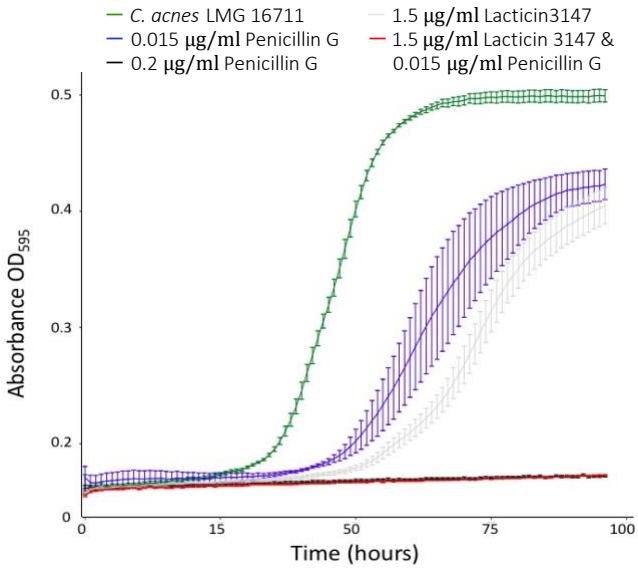
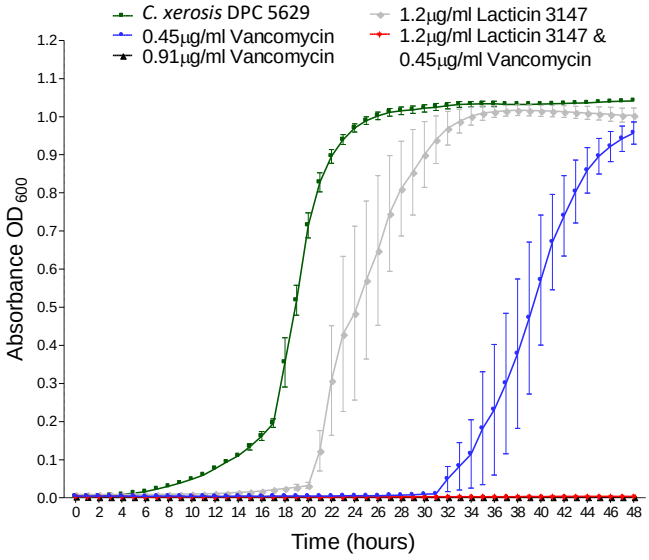


Figure 3.1 Deferred antagonism results of a bank of various bacteriocins producer strains against (A) *C. acnes* LMG 16711, (B) *Enterococcus faecium* UCC1, (C) *C. xerosis* DPC 5629 and (D) *Enterococcus faecium UCC2*. Deferred antagonism assays were carried out for MRSA ST291 but no zones of inhibition were observed. The zone of inhibition is expressed as the diameter of the zones minus the diameter of the 'spot' in mm. Blue bars indicate bacteriocins chosen for further analysis, lacticin 3147 and nisin Z.

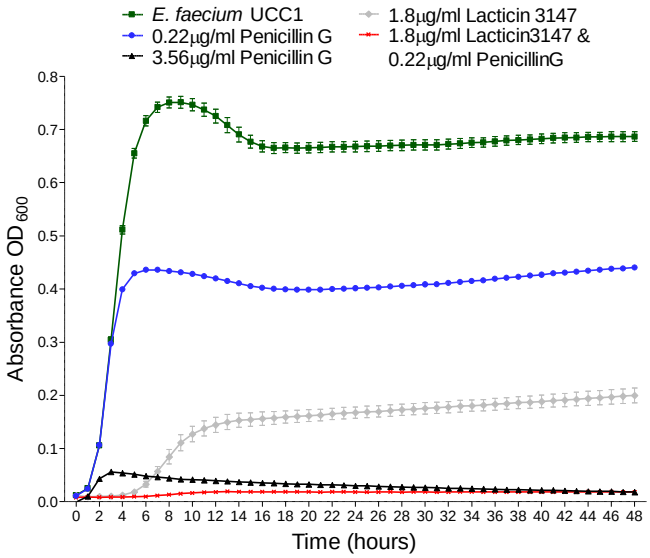
A


C. acnes LMG 16711
0.015 µg/ml Penicillin G
0.2 µg/ml Penicillin G
1.5 µg/ml Lacticin3147
1.5 µg/ml Lacticin 3147 &
0.015 µg/ml Penicillin G
Absorbance OD595
Time (hours)

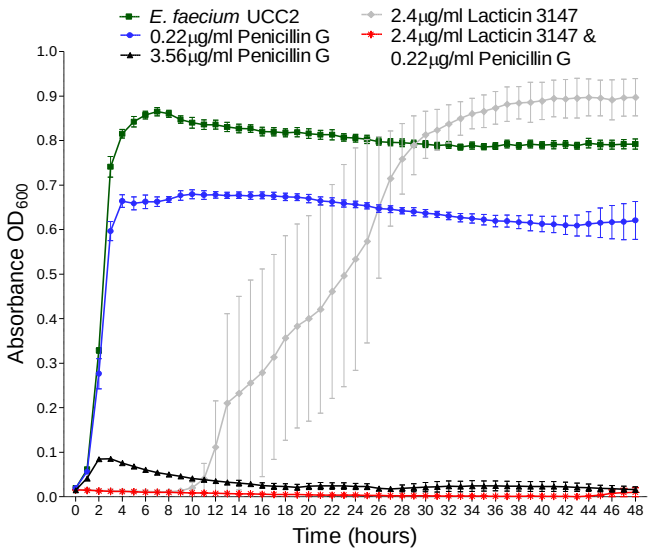
B


C. xerosis DPC 5629
0.45µg/ml Vancomycin
0.91µg/ml Vancomycin
1.2µg/ml Lacticin 3147
1.2µg/ml Lacticin 3147 &
0.45µg/ml Vancomycin
Absorbance OD600
Time (hours)

C


E. faecium UCC1
0.22µg/ml Penicillin G
3.56µg/ml Penicillin G
1.8µg/ml Lacticin 3147
1.8µg/ml Lacticin3147 &
0.22µg/ml PenicillinG
Absorbance OD600
Time (hours)

D



E

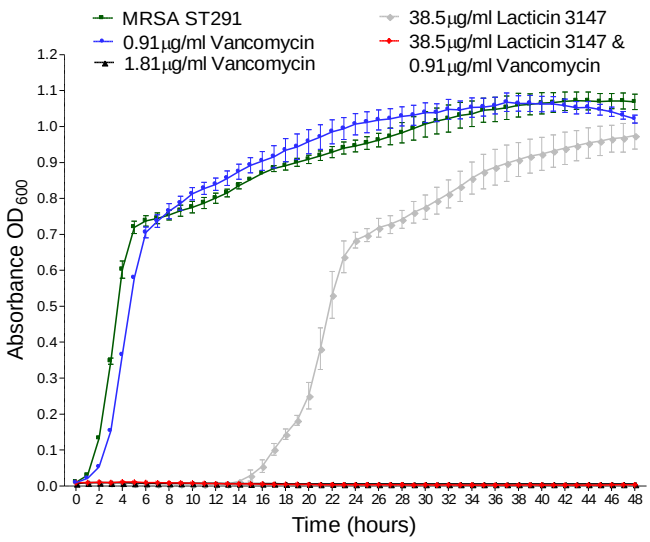
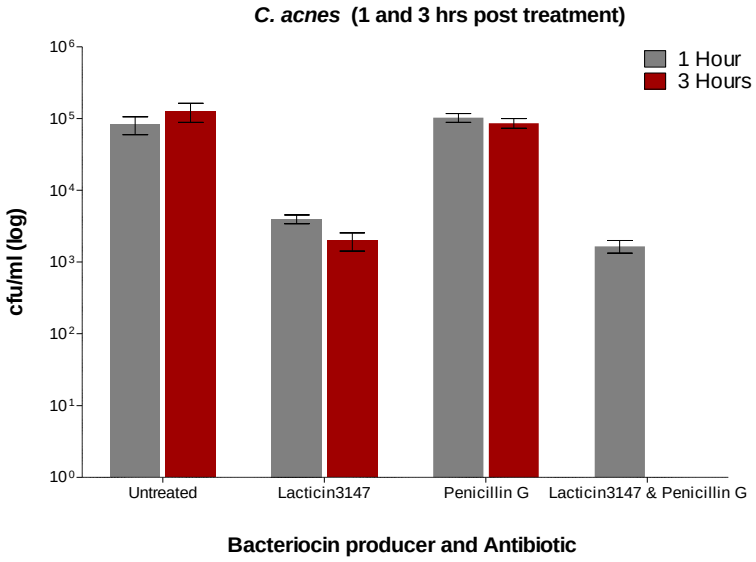
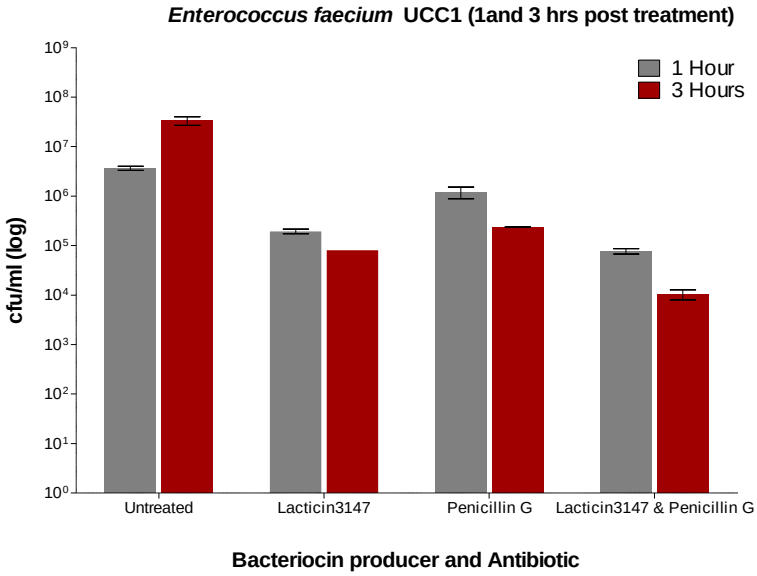


Figure 3.2 Growth curve results of (A) penicillin G (0.015µg/ml or 0.2µg/ml) and lacticin 3147 (1.5µg/ml) against *C. acnes* LMG 16711 (B) vancomycin (0.45µg/ml or 0.91µg/ml) and lacticin 3147 (1.2µg/ml) against *C. xerosis* DPC 5629, (C) penicillin G (0.22µg/ml or 3.56µg/ml) and lacticin 3147 (1.8µg/ml) against *E. faecium* UCC1, (D) penicillin G (0.22µg/ml or 3.56µg/ml) and lacticin 3147 (2.4µg/ml) against *E. faecium* UCC2 and (E) vancomycin (0.91µg/ml or 1.81µg/ml) and lacticin 3147 (38.5µg/ml) against MRSA ST291.

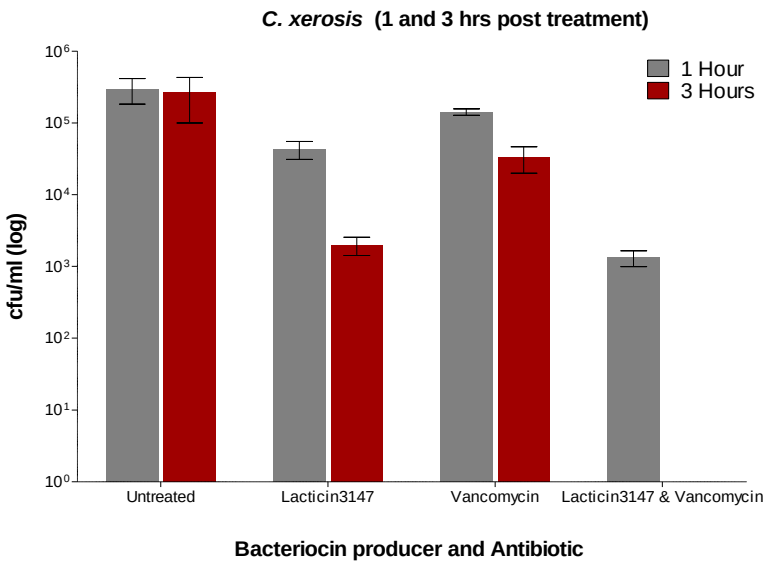
A


C. acnes (1 and 3 hrs post treatment)
1 Hour
3 Hours
cfu/ml (log)
Untreated
Lacticin3147
Penicillin G
Lacticin3147 & Penicillin G
Bacteriocin producer and Antibiotic

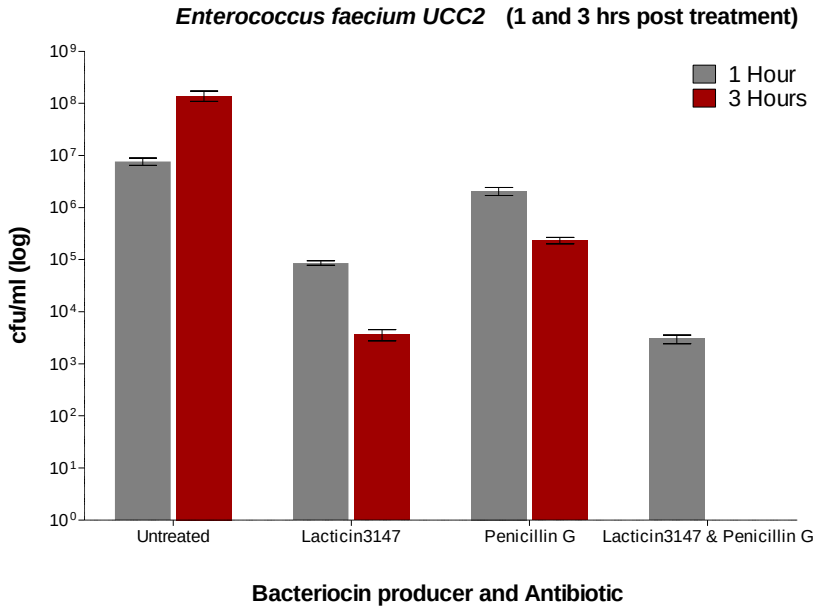
B


Enterococcus faecium UCC1 (1and 3 hrs post treatment)
1 Hour
3 Hours
cfu/ml (log)
Untreated
Lacticin3147
Penicillin G
Lacticin3147 & Penicillin G
Bacteriocin producer and Antibiotic

C


C. xerosis (1 and 3 hrs post treatment)
1 Hour
3 Hours
cfu/ml (log)
Untreated
Lacticin3147
Vancomycin
Lacticin3147 & Vancomycin
Bacteriocin producer and Antibiotic

D



E

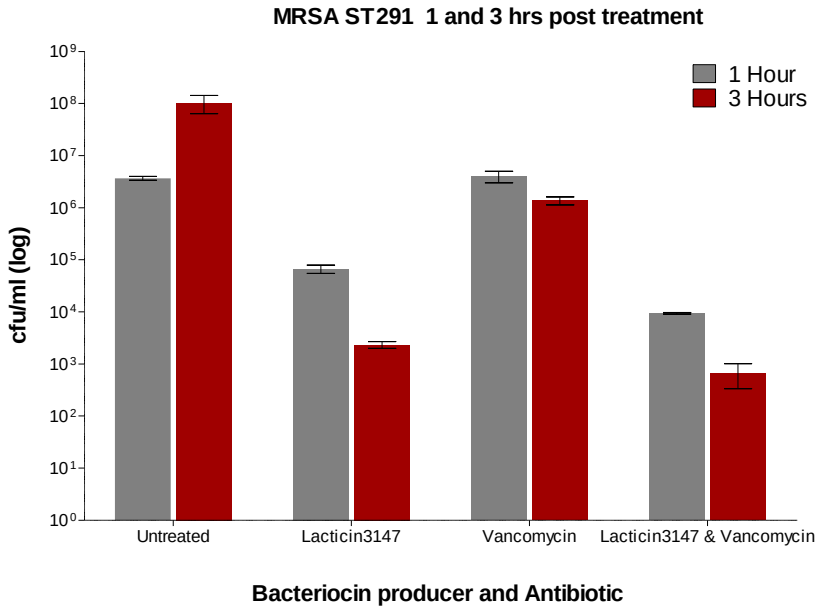
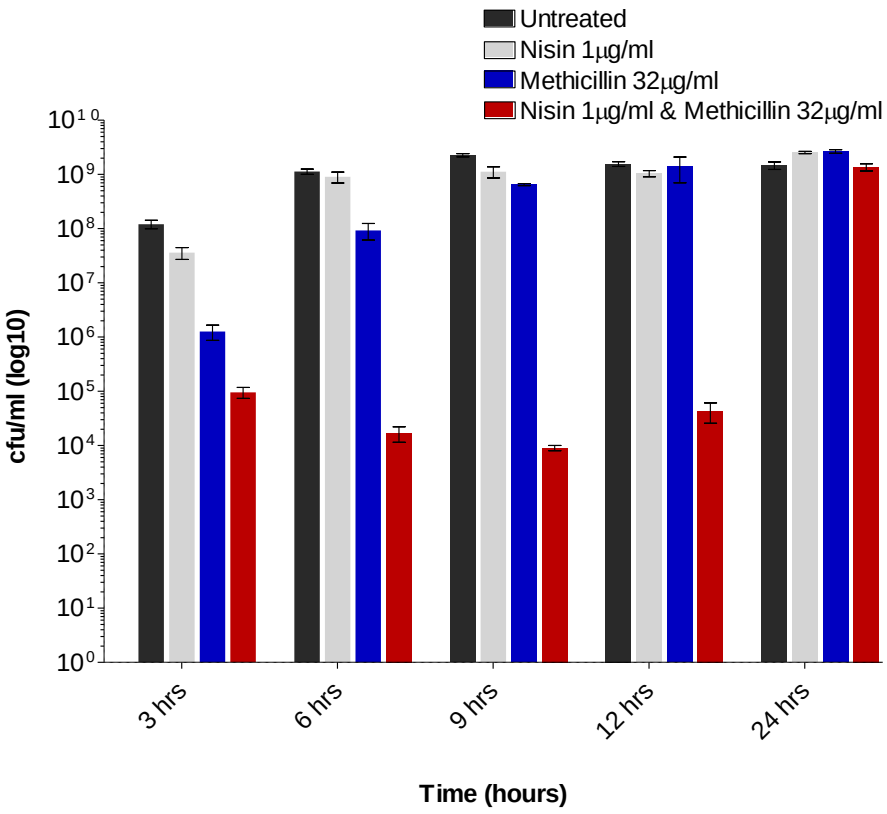


Figure 3.3 Time-kill results of (A) penicillin G (0.176µg/ml) and lacticin 3147 (7.5µg/ml) against *C. acnes* LMG 16711 (B) penicillin G (3.56µg/ml) and lacticin 3147 (1.80µg/ml) against *E. faecium* UCC1, (C) vancomycin (0.91µg/ml) and lacticin 3147 (1.2µg/ml) against *C. xerosis* DPC 5629, (D) penicillin G (3.56µg/ml) and lacticin 3147 (1.44µg/ml) against *E. faecium* UCC2 and (E) vancomycin (0.91µg/ml) and lacticin 3147 (11.55µg/ml) against MRSA ST291.

A



B

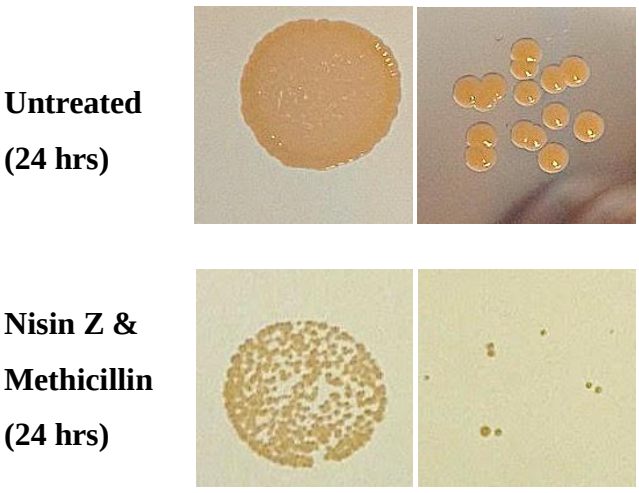


Figure 3.4 (A) Time-kill plate counts of MRSA ST291 3, 6, 9 and 12hrs post treatment with nisin Z (1μg/ml) and methicillin (32μg/ml). (B) Examination of MRSA ST291 colony morphology 24hrs untreated and post treatment with nisin Z and methicillin.

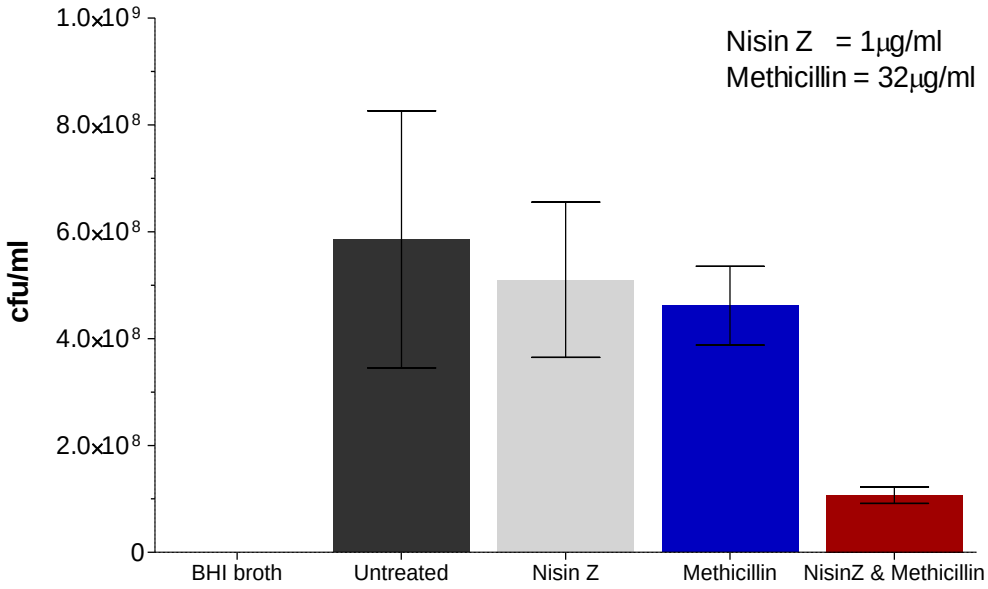


Figure 3.5 Ex vivo porcine skin model plate counts of MRSA ST291 3hrs post treatment with nisin Z (1µg/ml) and methicillin (32µg/ml).

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Chapter IV

Nisin Z induces apoptotic and autophagic cell death in colorectal and oesophageal cancer cell lines

3.1 Summary

In recent years the potential use of bacteriocins such as nisin as anti-cancer agents has received some attention. Nisin Z is a naturally occurring variant of nisin which has been safely used for many years as a food preservative. This study explored the anti-cancer effects of nisin Z on six different cell lines (HCT116, HT29, SW620, Caco2, OE19 and KYSE450). Nisin Z induced apoptotic cell death in all four colorectal cell lines and autophagic cell death in oesophageal cell lines. In addition, multiple primer sets were generated and qRT-PCR was used to examine the apoptotic and autophagic pathways of HCT116 and KYSE450. Apoptotic activity in nisin Z treated HCT116 cells was tightly controlled by means of upregulating caspase-9 (intrinsic pathway) along with the upregulating the anti-apoptotic Bcl-2 protein. In the case of nisin Z treated KYSE450 cells the upregulation of the Beclin gene confirmed autophagy induction. This data indicates that nisin Z induces programmed cell death by means of either apoptosis or autophagy in at least six difference cancer cell lines. Given its GRAS status nisin may well prove to be a viable alternative as a novel cancer therapeutic.

3.2 Introduction

With over 200 types and approximately 14.1 million new cases reported globally each year, it is not surprising that cancer is one of the most extensively researched areas in medicine[1]. Over the last few decades the number of global diagnoses has steadily increased and is expected to rise to 23.6 million new cases by the year 2030[1]. This increase is due to a number of factors which include a globally aging population, increasingly unhealthy lifestyles, tobacco smoking, and environmental factors among others[2–4]. In contrast to this, the number of cancer-related deaths has decreased from 49.72% to 36.39% (2015)[5]. According to the World Health Organisation (WHO), 1 in 6 mortalities are attributed to cancer, making it the second leading cause of death worldwide[6].

In 2018 9.6 million deaths were cancer related, with 862,000 of these due to colorectal cancer (8.98%)[6]. Adenocarcinomas account for approximately 96% of all colorectal cancers and affect the mucus forming cells which aid in the internal lubrication of the colon and rectum[7]. Although the survival rate has increased in recent decades, it remains the third most commonly diagnosed cancer in men and second in women[6,8–10]. The prognosis for a patient can range from a five year survival rate of as high as 92% to as low as 10%, depending on the cancer stage at the time of diagnosis[11,12]. Oesophageal cancer has the sixth highest mortality rate of all cancers and a five year survival rate of 40% to 11% depending on the stage[13]. The expected increase in incidence of colorectal cancer of up to 60% by the year 2030 and the relatively low survival rate for oesophageal cancer accentuates the need for improved or novel therapies[10].

Traditionally the methods used to treat cancer include chemotherapy, radiotherapy, chemo-radiotherapy and surgery. They are known to have a variety of side effects ranging in severity including, but not limited to, nausea/vomiting, hair loss, weight loss/gain, neutropenia,

stomatitis, diarrhoea, and aggravated insomnia[14–16]. This study aims to explore the idea of using bacteriocins as alternative therapeutics for the treatment of colorectal and oesophageal carcinomas.

Bacteriocins are antimicrobial peptides and have traditionally been used within the food industry as a food preservative. Recently nisin Z was shown to have a significant effect on floor-of-mouth oral cancer and head and neck squamous cell carcinoma (HNSCC) by reducing tumour volume and enhancing apoptosis, and blocking cell proliferation respectively, thus showing the potenitial for bacteriocins as a therapeutic for a number of cancers[17,18]. The theoretical therapeutic dose of nisin in humans was calculated at 66.7mg/kg body weight/day when converted from a 800mg/kg body weight/day dose in mice, which does not exceed the LD_{50} (lethal dose, 50%) reported in rodents[7–9]. When treated with 800mg/kg body weight/day for 3 weeks, tumour sizes in mice decreased by 5.8% with normal organ histology and no apparent inflammation, fibrosis or necrosis[18]. In addition, the FDA has approved the no-observed-adverse-effect-level (NOAEL) of <250ppm of nisin in food and the highest tested dose of nisin, 83.25mg/kg of diet, has been accepted[19].

Certain bacteriocins have demonstrated selective cytoxicity to various cancer cell lines which may be attributed to a number of factors. One such factor is that cancer cell membranes are known to have a predominantly negative charge due to an increase in the number of negatively charged phospholipids, such as phosphatidylserine, on the outer surface[21]. As bacteriocins are naturally positively charged they may preferentially bind to the negatively charged cancer cells over neutral healthy cells. Other factors include an increase in membrane fluidity of cancer cells thus allowing for membrane destabilization, and an increase in surface area on cancer cells due to a greater number of microvilli, allowing for more bacteriocin binding[21].

This potential new application of the well-studied nisin has attracted much interest, however its efficacy in cancers of the gastrointestinal tract has not yet been comprehensively evaluated. In this study, the potential efficacy of nisin was assessed as a treatment using six cell lines, derived from human tumours of the colon and oesophagus. Following treatment of the cells with nisin, pathways associated with apoptosis, autophagy and necrosis were analysed using a variety of experimental methods including morphological examination, flow cytometry, confocal microscopy and quantitative real-time polymerase chain reaction (qRT-PCR).

3.3 Experimental procedures

HCT116, HT29, SW620 and Caco2 cancer cell lines were acquired from ATCC, and OE19 and KYSE450 cell lines were from the UCC cell line collection. Cell lines grown in Dulbecco's Modified Eagle Medium (DMEM) were supplemented with 10% foetal calf serum (FCS). OE19 cells were grown in Rosewell Park Memorial Institute-1640 (RPMI) medium supplemented with 10% FCS and 1% L-glutamine. KYSE450 cells were grown in a 1:1 ratio of RPMI-1640 and Hams F12 nutrient mixture (HEPES modified without L-glutamine), supplemented with 10% FCS and 1% L-glutamine. All cell lines were incubated at 37°C in a humidified atmosphere with 5% CO_2 in air.

Cell line	Origin	Media
HT29	Colorectal adenocarcinoma	DMEM (supplemented with 10% FCS)
OE19	Oesophageal squamous cell carcinoma	RPMI-1640 (supplemented with 10% FCS and 1% L-glutamine)
HCT116	Colorectal carcinoma	DMEM (supplemented with 10% FCS)
Caco2	Colorectal adenocarcinoma	DMEM (supplemented with 10% FCS)
SW620	Colorectal adenocarcinoma	DMEM (supplemented with 10% FCS)
KYSE450	Oesophageal squamous cell carcinoma	1:1 RPMI -1640 & Hams F12 (supplemented with 10% FCS and 1% L-glutamine)

Table 4.1 Cell lines used in this study. DMEM (Sigma), RPMI-1640 without phenol red (Sigma), Hams F12 nutrient mixture (Sigma), and FCS (Sigma).

3.3.1 MTT assay

MTT is a colorimetric assay used to determine cell metabolic activity and thus, under defined conditions, cell viability. Metabolically active cells enzymatically reduce yellow tetrazolium to purple formazan, which can then be dissolved in DMSO in order to measure an absorbance reading.

At a cell density of $1x10^4$ cells per well were seeded into the wells of a 96 well flat-bottomed tissue culture plate (Sarstedt) and allowed to attach overnight at 37˚C in a humidified atmosphere with 5% CO_2. Following attachment, the cells were treated with either 800µg/ml nisin Z or nisin 1-28 (a truncated form of nisin shown to have 100-fold less activity against certain bacteria such as *L. Lactis*) (with DMEM as an untreated control) in triplicate and incubated at 37°C with 5% CO_2 for 96 hrs[22]. The media was subsequently removed by aspiration prior to adding 200µl of MTT (Thiazolyl Blue tetrazolium Bromide) (Sigma) solution (2mg/ml in serum free DMEM). Following 4hrs incubation (37˚C in a humidified atmosphere with 5% CO_2) the MTT solution was removed by aspiration and 200µl of DMSO added to each well ensuring homogeneity by gentle mixing. The absorbance at OD570nm was measured using a SpectraMax MP3 spectrophotometer and plotted as a precentage of untreated controls.

3.3.2 Colony Formation assay

Cells were seeded into a 24 well plate to a cell density of $1x10^5$ per well and allowed attach overnight at 37˚C in a humidified atmosphere with 5% CO_2. Cells were treated with 800µg/ml nisin Z (with DMEM as an untreated control) in duplicate and allowed incubate for either 72hrs or 96hrs. Following incubation the cells were harvested by collecting the supernatant and trypsinising attached cells, both of which were pooled. Cells were seeded to a

density of $2x10^3$ into a 6 well plate in triplicate and incubated until colonies were visible in the untreated control by macroscopic examination.

Following the removal of media by aspiration the cells were fixed with methanol for 10mins, washed with PBS and stained with Pro Diff II (Braidwood Laboratories) stain for a further 10mins. The cells were then gently rinsed with H_2O and allowed to dry before photographing. Colonies were enumerated using ImageJ software and colony numbers of treated cells were plotted as a percentage of untreated cells.

3.3.3 Preparation of cells for morphological examination using cytospin

Cells were seeded into a 6 well plate at a density of $5x10^5$ cells per well in triplicate and allowed attach overnight at 37˚C in a humidified atmosphere with 5% CO_2. Following incubation cells were treated with either 600µg/ml or 800µg/ml nisin Z (with DMEM as an untreated control) in duplicate and incubated for 72hrs or 96hrs. Following incubation the cells were harvested by collecting the supernatant and trypsinising attached cells. Approximately 10,000 cells were spun onto glass slides (Cytopsin 4, Thermo Scientific) at 400rpm for 2mins. The cells were then fixed with methanol for 5mins and stained with Pro-Diff (Braidwood Laboratories). Cells were analysed using light microscopy at 40X.

3.3.4 Flow cytometry

The percentage of apoptotic cells induced by nisin Z treatment was determined by flow cytometry. Cells were seeded into a 24 well plate at a cell density of $5x10^4$ cells per well in triplicate and allowed attach overnight at 37˚C in a humidified atmosphere with 5% CO_2. Following incubation the cells were treated with 0.05mM 5-FU (positive control) or 800µg/ml nisin Z (with DMEM as an untreated control) and incubated for 96hrs. Following incubation the cells were harvested by collecting the supernatant and trypsinising the remaining attached

cells. Cells were pelleted by centrifugation at 130rcf for 10mins and resuspended in 500µl 1X binding buffer followed by the addition of 5µl of propidium iodide (PI) (50µg/ml) and 5µl of Annexin V-FITC (Apoptosis Detection Kit, Abcam) before incubation at room temperature for 5mins in the dark. The suspensions were transferred to plastic cytometry tubes for analysis by flow cytometry. Samples were analysed using a BD Celesta Fluorescence Activated Cell Sorter (FACS). 10,000 events were analysed per sample using the 488 (blue) laser, for FITC an excitation/emission of 494/519nm was used and 493/636nm for PI. Resulting data was analysed using FACS Diva software. Gating limits (set up to exclude cellular debris), laser position, side scatter and forward scatter plots were established first by analysing untreated controls. Test samples were then analysed using these parameters.

3.3.5 Confocal microscopy

Cells to be assayed were seeded into a a 24 well plate at a density of $5x10^4$ per well in triplicate and allowed attach overnight at 37˚C in a humidified atmosphere with 5% CO_2. Following incubation the cells were treated with either 6.5g/L 5-FU (positive control) or 800µg/ml nisin Z (with sterile DMEM as an untreated control) and subsequently incubated (37˚C in a humidified atmosphere with 5% CO_2) for 72hrs. Following incubation, the cells were harvested by collecting the supernatant and trypsinising the remaining attached cells. Cells were prepared for live cell analysis by confocal microscopy using CYTO-ID green detection reagent and Hoechst 33342 nuclear stain (CYTO-ID autophagy detection kit, Enzo). Following incubation at (37˚C in a humidified atmosphere with 5% CO_2) for 30mins the cells were pelleted by centrifugation at 100rcf for 10mins and resuspended in 100µl assay buffer. Using a 63X lens on a laser scanning microscope (LSM) 5 exciter (Zeiss LSM 5 Exciter, Zeiss, Germany), the cells were imaged using both an Argon LGK 7812 Lasos laser (fluorescence detected at wavelength 488nm, 25.0mW) and Diode 405-25 NT405 Carl Zeiss laser

(fluorescence detected at wavelength 405nm, 25.0mW). Images were analysed using Zen2008 software.

3.3.6 qRT-PCR

Cells to be assayed were seeded into a 24 well plate at a density of $1x10^5$ per well in triplicate and allowed attach overnight at 37˚C in a humidified atmosphere with 5% CO_2. Following incubation the cells were treated with 800µg/ml nisin Z (with sterile DMEM as a negative control) and incubated (37˚C in a humidified atmosphere with 5% CO_2) for 96hrs. Following incubation the cells were harvested by collecting the supernatant and trypsinising the remaining attached cells. RNA was extracted from the cells (Rneasy mini kit, Qiagen) before checking the quality (2100 Bioanalyser system, Agilent) and quantity (Qbit fluorometric RNA HS assay kit, Invitrogen™) of each RNA sample. Each sample was converted into cDNA using Reverse Transcription PCR (RT-PCR). RT-PCR was executed with a total of 20µl comprised of 4µl 5X transcriptor buffer (Roche), 2µl dNTP nucleotide mix (10mM) (Roche), 1µl Transcriptor Reverse Transcriptase and Protector Rnase inhibitor mix (Roche), 3µl Random primer $p(dN)_6$ (Roche) and 10µl RNA sample. Cycling conditions were as follows: 25°C for 10mins, 55°C for 30mins and 85°C for 5mins. Samples were then diluted using 80µl PCR-grade H_2O and 20µl of cDNA sample.

Quantitative real-time PCR (qRT-PCR) analysis was performed on each sample using a LightCycler 480 (Roche) machine and software (release 1.5.0). For the PCR reaction a total of 10µl comprising of 5µl Sybr green (2X SensiFAST SYBR Hi-ROX Kit, Labgene), 0.5µl of both forward and reverse primers (10µM) (Table 4.2) (Sigma Aldrich), 1µl PCR-grade H_2O and 3µl cDNA sample was used. qRT-PCR conditions were as follows: pre-incubation cycle of 50°C for 2mins followed by 45 cycles of 95°C for 10secs, 60°C for 45secs and 72°C for

1sec and a melting temperature of 95°C. The endogenous expression of beta actin was used as an internal reference. Data normality was determined using a Shapiro-Wilk test and futher analysis was performed using a paired t-test.

3.4 Results

3.4.1 MTT assay

An MTT assay performed on KYSE450 and HCT116 cell lines 96hrs post-treatment with 800μg/ml nisin 1-28 (shown to be up to 100-fold less active against various bacterial strains) showed almost no effect on survival with a % survival >80% in the case of both cell lines (Figure 4.1). This is in contrast to the effects obseved when treated with nisin Z, with significant decreases in survival with a cell survival of <50% being recoreded in the case of both cell lines (Figure 4.1).

3.4.2 Colony Formation assay

Cell recovery was measured for each cell line by means of colony formation assays of morphologically intact cells (Figures 4.2, 4.3). Recovery levels decreased in all six cell lines treated with either 600μg/ml or 800μg/ml of nisin Z at both 72 and 96hrs, compared to an untreated control. Four of the six cell lines demonstrated a significant decrease in recovery at 96hrs post-treatment, two at a concentration of 800μg/ml (KYSE450 and SW620) and two showed a marked decrease in recovery at the lower concentration of 600μg/ml (OE19 and HT29). HCT116 cells were more sensitive to nisin Z at a concentration of 600μg/ml 72hrs post treatment than when treated for 96hrs. Recovery levels of Caco2 cells were lower when treated with nisin Z at a concentration of 800μg/ml than 600μg/ml, however, recovery levels 96hrs post treatment were comparable to 72hrs of treatment. Of all the cell lines, KYSE450 proved to be the most sensitive to nisin Z treatment (800μg/ml), as evidenced by a significant decrease in colony count following staining at both 72 and 96hrs (Figures 4.2 A, B, 4.3 C)

3.4.3 Morphological examination

Post treatment with nisin Z (600μg/ml and 800μg/ml) for both 72 and 96hrs, cells were prepared via cytospinning, for morphological examination using microscopy techniques. Membrane blebbing in HT29, SW620, Caco2 and HCT116 cell lines (Figures 4.4 A, C, D, F), and autophagic vacuoles in the cytoplasm of OE19 and KYSE450 cells lines (Figures 4.4 B, E) were observed. Membrane blebbing or autophagic vacuoles were observed in each cell line at both time points (72 and 96hrs of treatment) and with both concentrations of nisin Z (600μg/ml and 800μg/ml).

3.4.4 Flow cytometry

Flow cytometry was performed on nisin Z treated cells (800μg/ml for 96hrs) to determine the mode of cell death. Healthy cells predominantly occupy the lower left quadrant of the scatter plot, indicating that they haven't bound PI or FITC-tagged AnnexinV. FITC-tagged AnnexinV will bind early apoptotic and late apoptotic cells, with late apoptotic cells also binding PI. Cell stained only with PI are considered to be necrotic.

Nisin Z treated CACO2 cells contain an increased population of PI+AnnexinV+ and AnnexinV+ cells compared to an untreated control, 34.8% and 3.8% respectively (Figure 4.5 D), suggesting an apoptotic mode of cell death.

In the case of HCT116, when compared to untreated control, Nisin Z-treated cell populations presented as late apoptotic (30.4%) with a smaller population showing as early apoptotic (4.7%) (Figure 4.5 B). Nisin Z (800μg/ml) treated SW620 cells display a similar shift towards both late (66.0%) and early (20.1%) apoptosis; even more so than the 5-FU treated cells of 56.4% and 15.3% respectively (Figure 4.5 C).

In comparison to the untreated population, the majority of nisin Z (800μg/ml) treated KYSE450 cells present as necrotic (14.6%) and late apoptotic (49.2%) (Figure 4.5 F). A

similar trend was seen in the FACS analysis of Nisin Z-treated OE19 cells. 9.7% of the population of treated cells presented as necrotic and 27.3% presenting as late apoptotic (Figure 4.5 E). The majority of nisin Z (800μg/ml) treated HT29 cells, when compared to untreated cells, present as late (22.4%) and early apoptotic (12.7%) (Figure 4.5 A).

3.4.5 Confocal microscopy examination

Confocal laser scanning microscopy (CLSM) was used to image autophagic vacuoles of nisin Z (800μg/ml) treated KYSE450 and OE19 cell lines (Figure 4.6). Hoechst 33342 nuclear stain, which preferentially binds to adenine-thymine regions of eukaryotic DNA, was used for visualising cell nucleoli (blue fluorescence), whereas CYTO-ID green detection reagent was used to identify the accumulation of autophagic vacuoles through specific labelling (yellow fluorescence). Distinctive autophagic vacuoles accumulated in nisin Z (800μg/ml) treated KYSE450 and OE19 cell lines when compared to untreated control (Figure 4.6). Autophagic vacuoles can also be seen in 5-FU (positive control) treated cell.

3.4.6 qRT-PCR

qRT-PCR analysis was employed to determine the apoptotic and autophagic pathways taken by HCT116 and KYSE450 cells lines. In the case of nisin Z (800μg/ml) treated HCT116 cells caspase-9 and Bcl-2 were both upregulated when compared to the untreated control, whereas there was no statistically significant change in the expression levels of CHOP, caspase-8 and caspase-12. Nisin Z (800μg/ml) treated KYSE450 cells, showed upregulation of beclin expression, whereas CHOP and BNIP3 were down regulated.

3.5 Discussion

Traditionally nisin has been used as a food preservative, but recently it has become the subject of much interest with regards to its use as a therapeutic against a range of clinically significant bacteria, and more recently for its potential as a therapeutic for certain cancers[17,18,23]. In particular HNSCC cell lines showed a significant reduction in tumour volume, enhanced apoptosis and blocked cell proliferation, when treated with nisin Z[17,18]. Here we investigate whether the therapeutic effects of nisin can extend to a panel of oesophageal and colorectal cancer cells lines, namely HCT116, SW620, HT29 Caco2, KYSE450 and OE19 (Table 4.1).

Initial MTT assays were performed on two different cell lines to determine the effect of nisin Z on each cell line in comparison to the nisin variant nisin 1-28, which is known to be 100-fold less active against certain bacteria[22]. A significant decrease in cell survival was seen in cells treated with nisin Z in comparison to cells treated with nisin 1-28 (Figure 4.1). This suggests that the truncated variant's (nisin 1-28) activity against selected cell lines (KYSE450 and HCT116) is also decreased, along with the specific structure of nisin Z playing a role in how effective the peptide is against these specific cancer cells lines.

Colony formation assays were employed to determine the ability of each cell line to recover from nisin Z treatment, using both 600μg/ml and 800μg/ml, at two different time points (72 and 96hrs). A notable degree of variability was seen between each cell line in terms of recovery after 96hrs of treatment, but less so at 72hrs of treatment (Figure 4.2). KYSE450 and OE19 cell lines have previously been shown to induce autophagy when stressed[24]. Autophagy is a highly conserved survival response to unfavourable conditions, in which cellular components are degraded and recycled and is genetically regulated by a group of genes called the ATG genes. Autophagy can play a role in achieving a homeostatic state in normal cells, but

it has previously been shown to aid cancer cell survival in the presence of chemotherapeutic drugs, including 5-FU treated KYSE450 and OE19 cell lines[24]. In contrast to this our results display an increased level of autophagy but a decrease in percentage recovery of nisin Z treated KYSE450 and OE19 cell lines. Out of the six cell lines tested KYSE450 showed the lowest rate of recovery and OE19 the third lowest rate of recovery, thus provoking the idea that nisin Z stimulates an autophagy-linked type II programmed cell death (PCD). Previous studies have shown that autophagy alone may be able to induce cell death (type II PCD), however other studies have shown that autophagy more commonly increases dramatically before cell death by means of apoptosis (type I PCD), leading to the question of whether nisin Z causes cell death by autophagy alone or apoptosis, or both. Regulation of autophagy and apoptosis has been shown to overlap, with cells either positively or negatively stimulating the interactions between the anti-apoptotic protein Bcl-2 and the autophagy protein Beclin 1[25]. In nutrient-rich condition Bcl-2 can inhibit the autophagic activity of Beclin 1 thereby inhibiting autophagy; this interaction can also be blocked, thus allowing for autophagic activity[25].

Preliminary morphological examination determined the mode of cell death following treatment with nisin Z for both 72 and 96hrs (600μg/ml and 800μg/ml). This revealed that nisin-treated HT29, Caco2, SW620 and HCT116 cells lines had obvious cytoplasmic membrane blebbing along with some chromatin condensation, which are both morphologically distinguishable features of apoptotic cells (Figures 4.4 A, C, D, F). Cell lines OE19 and KYSE450 had numerous vacuoles within the cytoplasm when compared to untreated control, indicating autophagic activity (Figures 4.4 B, E). To further determine mode of cell death PI and Annexin V FITC stained cells were analysed by flow cytometry. PI was used to distinguished necrotic cells, whereas Annexin V conjugated to green-fluorescent FITC dye was used to identify apoptotic cells by detecting phosphatidylserine that is exposed on the cell surface of apoptotic cells. SW620 cells gave the most striking results with 21.1% of cells

appearing as early apoptotic and 66% as late apoptotic, which was a greater number than the 5-FU treated positive control. For treated HT29 cells 35.1% were either early or late apoptotic. Consistent with the colony formation assay results, SW620 and KYSE450 cell lines had the fewest number of cells remaining in the healthy quadrant, 13.1% and 33.1% respectively. HT29, HCT116, SW620 and Caco2 cells lines show results consistent with apoptotic mode of cell death. In the case of KYSE450 cell line, nisin Z treated cells did not display a strong apoptotic profile despite the high level of cell death observed in other assays (Figure 4.5). KYSE450 showed a high sensitivity to nisin Z in the clonogenic assays, and displayed an abundance of cytoplasmic vacuoles in morphological assays and a weak apoptotic profile when examined by flow cytometry. It is possible that KYSE450 cells are dying by means of autophagy or type II PCD. OE19 cell lines had a high level of cells present as apoptotic. With morphological examination clearly showing cytoplasmic vacuoles, it is possible that cells are under extreme stress and are producing vacuoles for attempted survival before dying by means of apoptosis. It has been shown in previous studies that some cell lines, before dying by apoptosis produce vast amount of vacuoles in an attempt to undergo autophagy recycling in order to survive[25].

Autophagic vacuoles were distinguished from accumulated lysosomes using confocal laser scanning microscopy (CLSM), further establishing autophagic activity within OE19 and KYSE450 cell lines. Nisin Z and 5-FU treated cells were stained with CYTO-ID green detection reagent and Hoechst 33342 nuclear stain (CYTO-ID autophagy detection kit, Enzo). Both OE19 and KYSE450 display autophagic vacuoles when compared to the untreated control (Figure 4.6). In the untreated controls, there is either a complete absence or significantly fewer numbers of autophagic vacuoles. A small number of autophagic vacuoles is normal within healthy cells as they can play a part in maintaining population homeostasis as mentioned

previously[24]. However, in 5-FU or nisin Z treated cells there is an obvious increase in the number of accumulated autophagic vacuoles, suggesting acute cell stress.

qRT-PCR was used to determine the apoptotic and autophagic pathways taken by HCT116 and KYSE450 cell lines. Chemotherapy drugs have been shown to activate various different pathways within cancer cells leading to apoptosis, such as the intrinsic and/or extrinsic pathways, and a failure to do so may lead to treatment resistance[26,27]. Caspases, important apoptosis effector molecules within the extrinsic and intrinsic pathways, can be activated at either a specific receptor or mitochondria, both ultimately initiating a caspase cascade leading to apoptosis[27]. Caspase-8, involved in the extrinsic pathway, can be activated by the stimulation of receptors, such as Fas and TRAIL (Figure 4.8), resulting in the activation of apoptosis through the cleavage of the downstream effectors such as caspase-3[27]. Caspase-9 can also activate caspase-3 to induce apoptosis by means of the intrinsic pathway. This entails the release of an apoptogenic factor, such as cytochrome *c* which when released into the cytosol forms an apoptosome complex with Apaf-1 and caspase-9 (Figure 4.8)[27]. Endoplasmic reticulum stress can also be involved in multiple apoptotic pathways, including an unfolded protein response (UPR) pathway and a caspase-12 involved pathway (Figure 4.8)[27,28].

In the case of nisin Z treated HCT116 cells, the transcription of the caspase-9 gene involved in the intrinsic pathway, was upregulated in addition to the anti-apoptosis protein, Bcl-2. None of the other genes involved in the extrinsic or UPR pathways were upregulated, suggesting that nisin Z induced an intrinsic apoptosis pathway in HCT116 cells. Due to the colony formation assay, macroscopy and flow cytometry results displaying partial apoptotic activity, and the upregulation of the protein caspase-9, the anti-apoptosis protein Bcl-2 seems to be playing an active role in protecting the cells from nisin Z-induced apoptosis.

The protein Beclin, previously shown to be associated with the induction of autophagy cell death in KYSE170 cells, was also upregulated in nisin Z treated KYSE450 cells[29].

However, the autophagy associated protein BNIP3 was significantly down regulated in KYSE450 cells, indicating that an alternative mechanism must have been adopted to achieve autophagy than in the case of ethyl-3,4-dihydroxybenzoate treated KYSE170 cells[29]. KYSE450 cell lines have also previously been shown to enter an acute state of autophagy before initiating apoptotic cell death, therefore it is still unclear whether nisin Z treated KYSE450 cells experience a heightened state of autophagy before ultimately being killed by apoptosis or die from autophagy alone.

In recent years bacteriocins such as nisin have been shown to produce cytotoxicity in various cancer cell lines both *in vitro* and *in vivo*[29]. With an enhanced expression of negatively charged molecules on their cell surface, cancer cells can be susceptible to cytotoxic activity produced by positively charged bacteriocins[21,30]. In this study nisin Z demonstrates *in vivo* cytotoxic activity in the form of either apoptosis or autophagy in six different cancer cell lines of either the esophagus or colon. As nisin has already been approved by the FDA and WHO for human consumption, along with its non-immunogenic nature and promising *in vitro* and *in vivo* anti-cancer results, it's use alongside conventional anti-cancer drugs should be evaluated as a step further towards its potential use as a viable therapeutic[29].

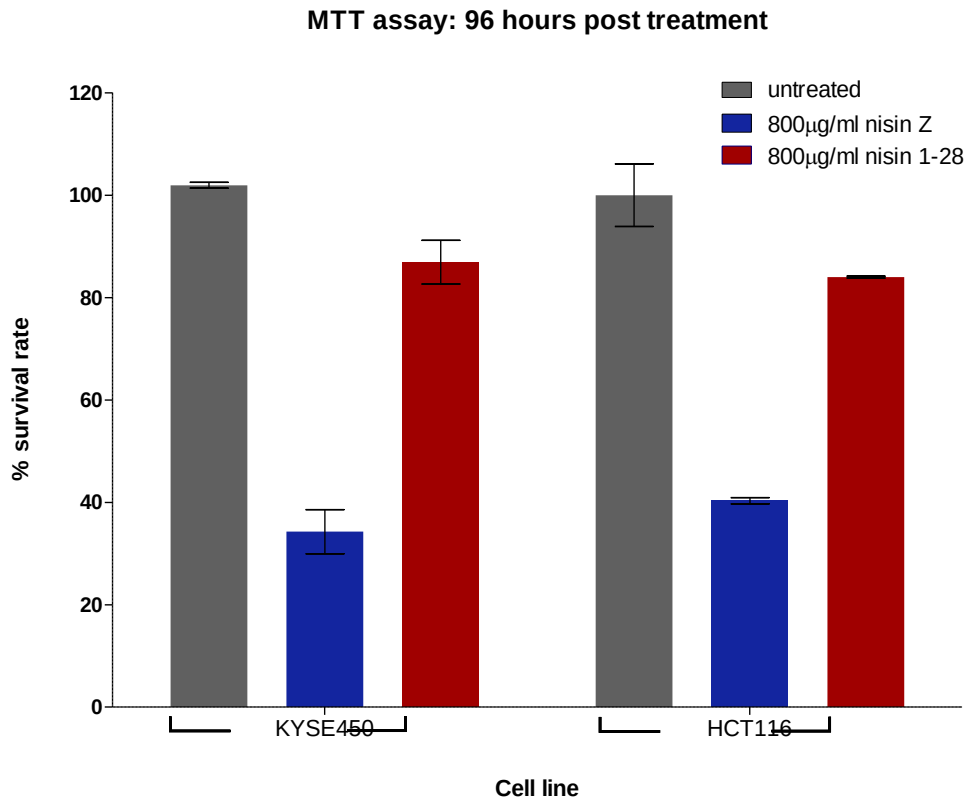
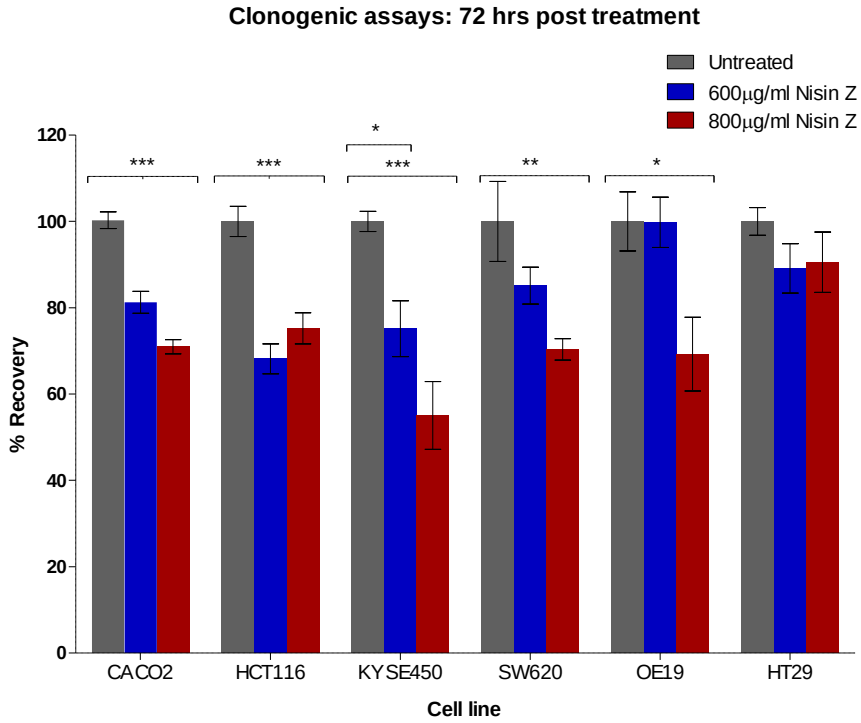


Figure 4.1 MTT % survival results of cell lines KYSE450 and HCT116 untreated and treated with both 800µg/ml of nisin Z and nisin 1-28 for 96hrs.

A



B

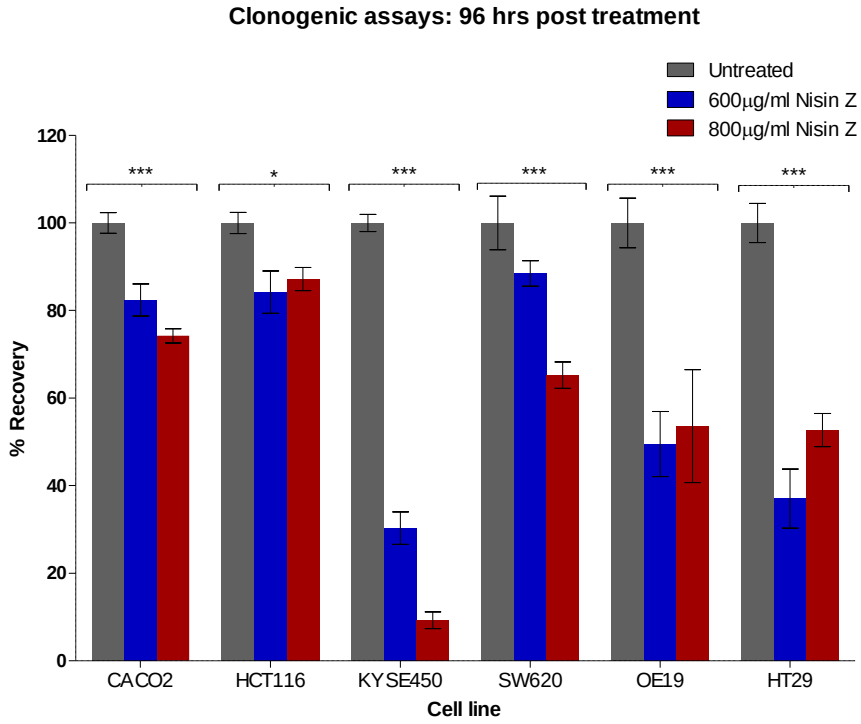


Figure 4.2 Colony Formation assay results of cell lines Caco2, HCT116, HT29, KYSE450, OE19 and SW620 treated with both 600µg/ml and 800µg/ml of nisin Z for (A) 72hrs and (B) 96hrs.

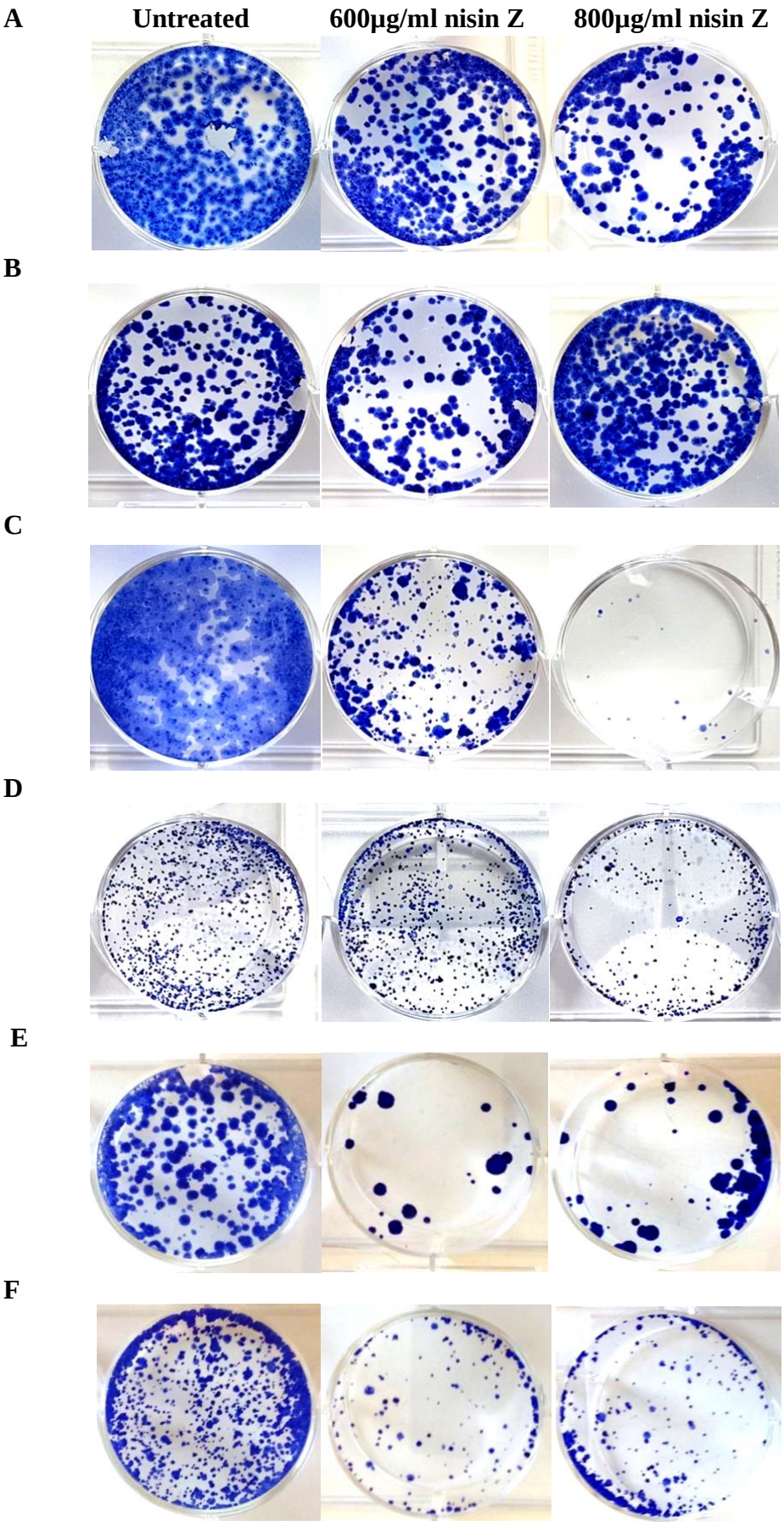
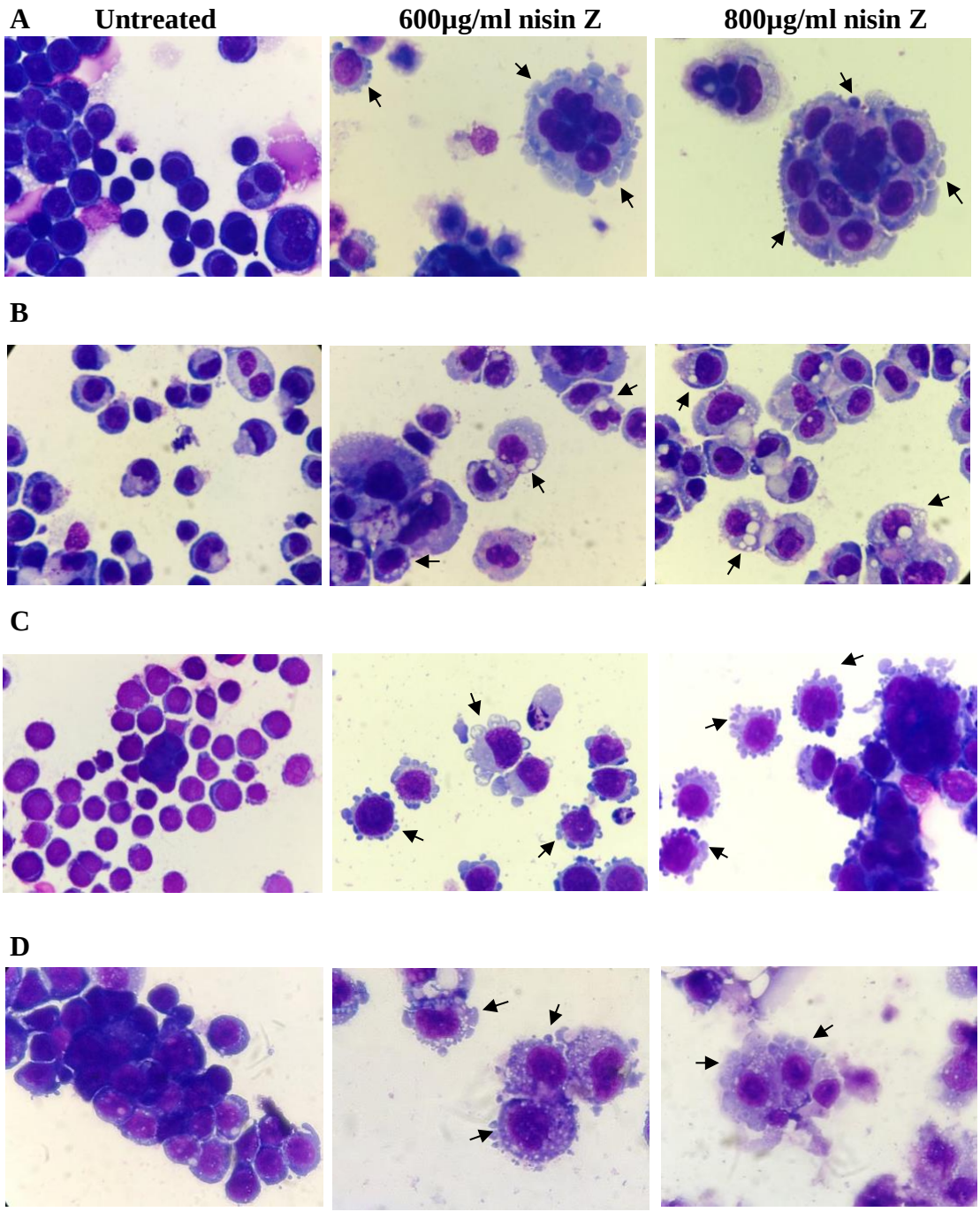

A
Untreated
600µg/ml nisin Z
800µg/ml nisin Z
B
C
D
E
F

Figure 4.3 Colonies formations of (A) Caco2, (B) HCT116, (C) KYSE450, (D) SW620, (E) OE19 and (F) HT29 cell lines 96hrs post treatment with 600µg/ml and 800µg/ml nisin Z.


A
Untreated
600µg/ml nisin Z
800µg/ml nisin Z
B
C
D

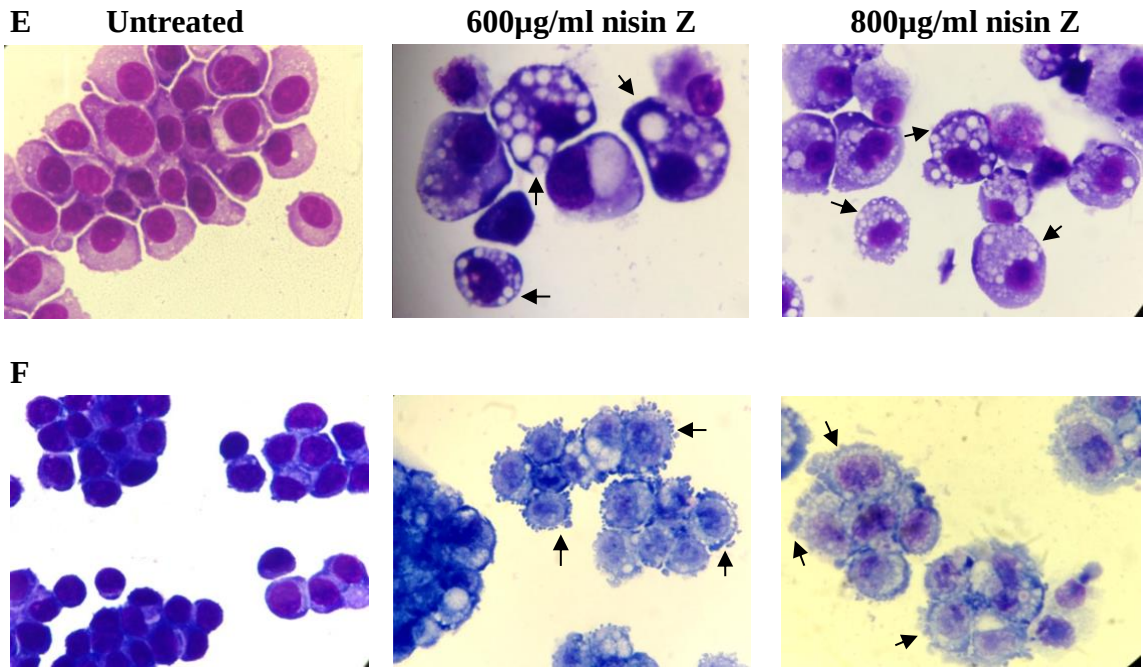
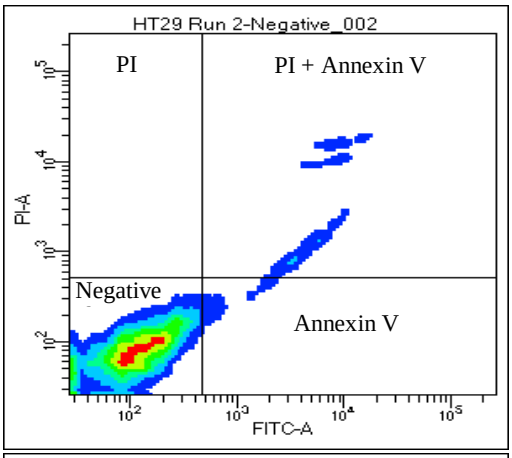
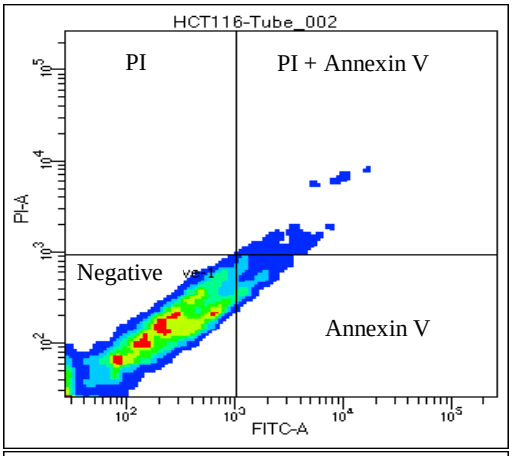


Figure 4.4 Cytospin results of: (A) HT29, (B) OE19, (C) SW620 and (D) Caco2 cell lines 96hrs post treatment, and (E) KYSE450 and (F) HCT116 cell lines 72hrs post treatment. Figures represent untreated and treated cell with 600µg/ml nisin Z and 800µg/ml nisin Z for all cell lines. In the cases of (A), (C), (D) and (F) arrows indicate cytoplasmic blebbing, and in the case of (B) and (E) arrows indicate autophagic vacuoles.

A **HT29** **B** **HCT116**

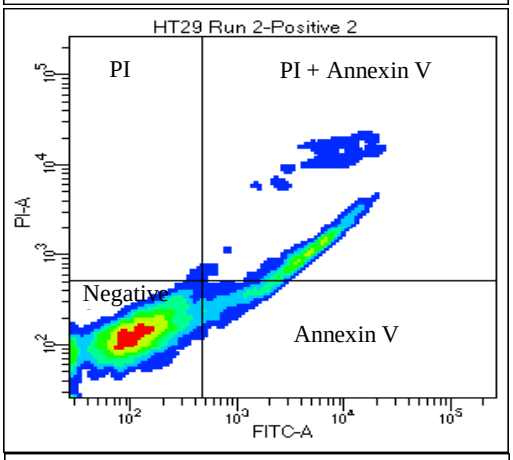
Untreated

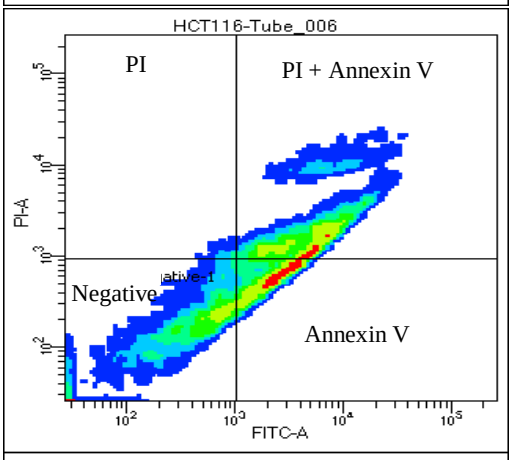




0.05mM

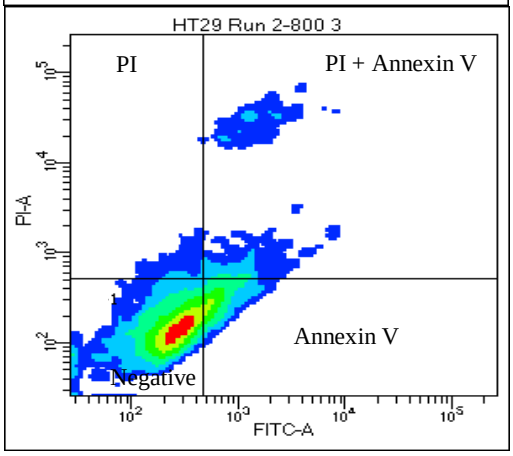
5-FU

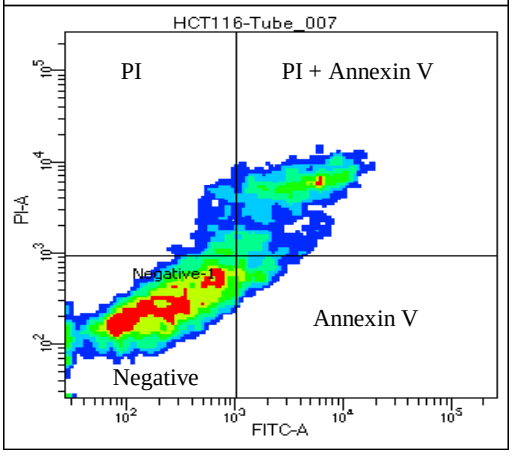




800µg/ml

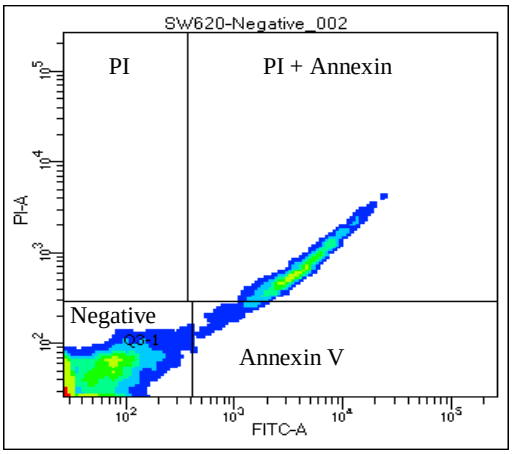
Nisin Z

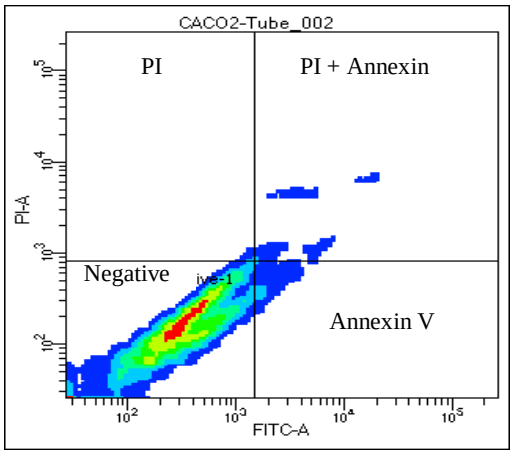




C **SW620** **D** **Caco2**

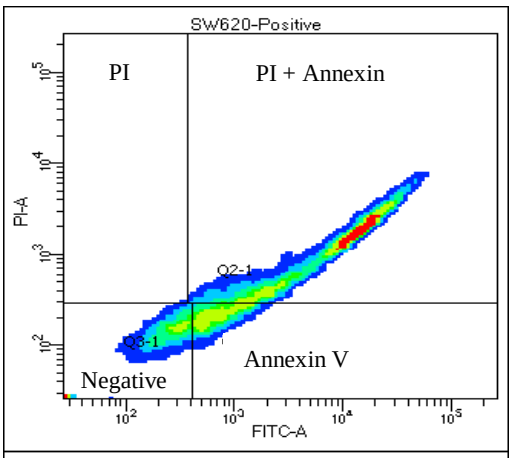
Untreated

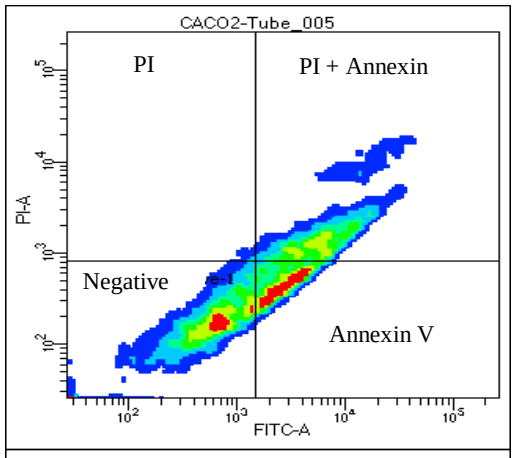




0.05mM

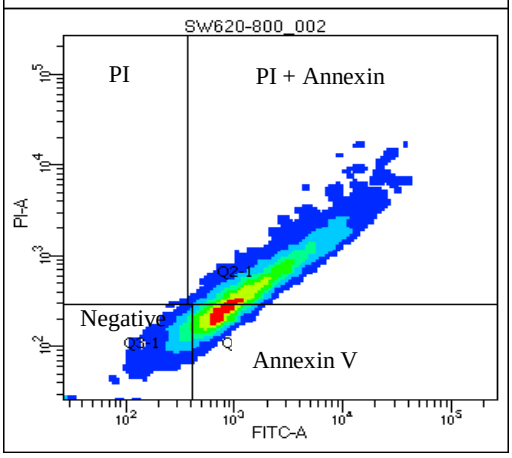
5-FU

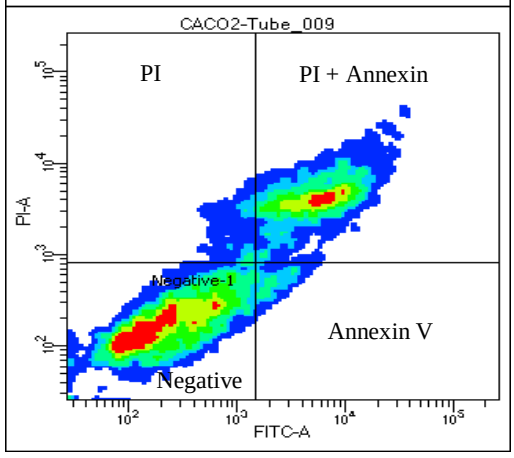




800µg/ml

Nisin Z





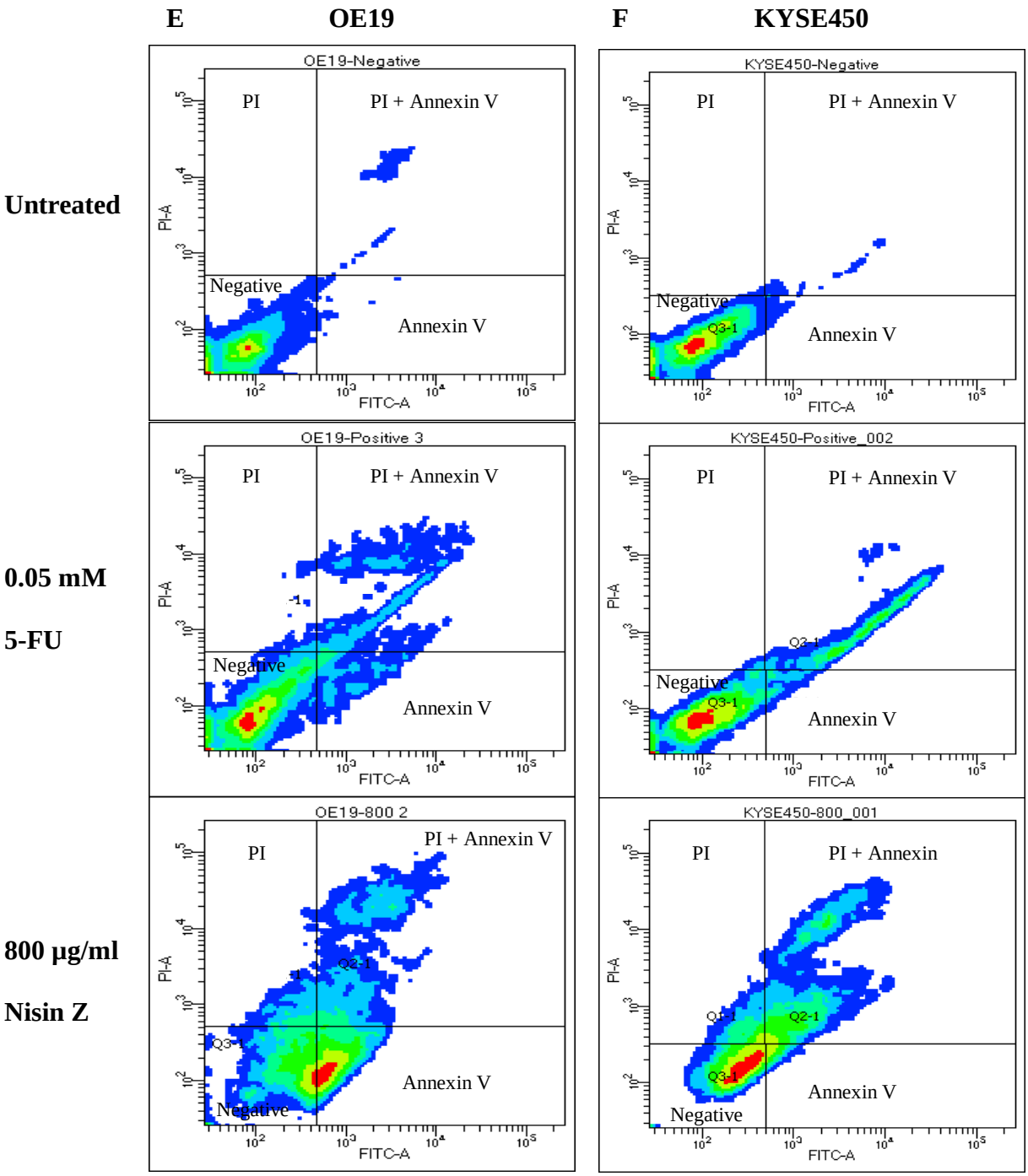


Figure 4.5 Flow cytometry scatter profile of (A) HT29, (B) HCT116, (C) SW620, (D) Caco2, (E) OE19 and (F) KYSE450 cells untreated and 96hrs post treatment with 0.05mM 5-FU and 800µg/ml nisin Z.

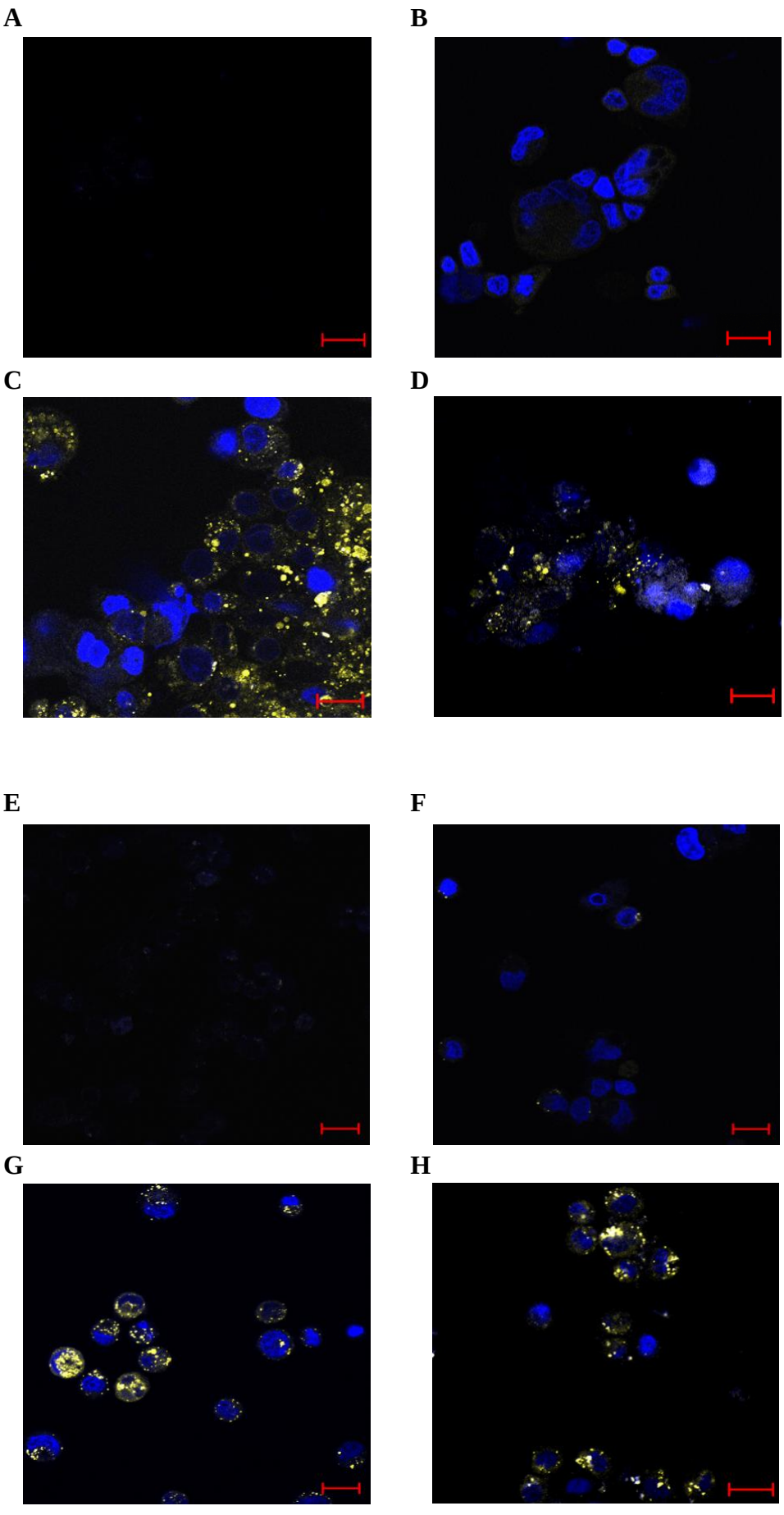
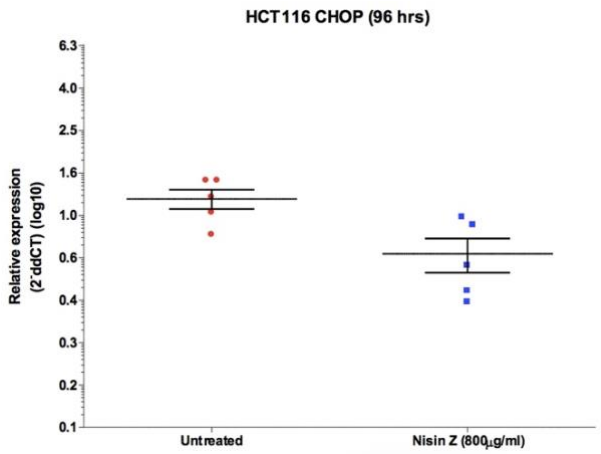

A
B
C
D
E
F
G
H

Figure 4.6 Confocal microscopy results of KYSE450 cells at 72hrs post treatment, represented by (A) unlabelled, (B) untreated, (C) treated with 0.05mM 5-FU and (D) treated with 800µg/ml nisin Z. Confocal microscopy results of OE19 cells at 72hrs post treatment represented by (E) unlabelled, (F) untreated, (G) treated with 0.05mM 5-FU and (H) treated with 800µg/ml nisin Z. Ruler indicates 20µm.

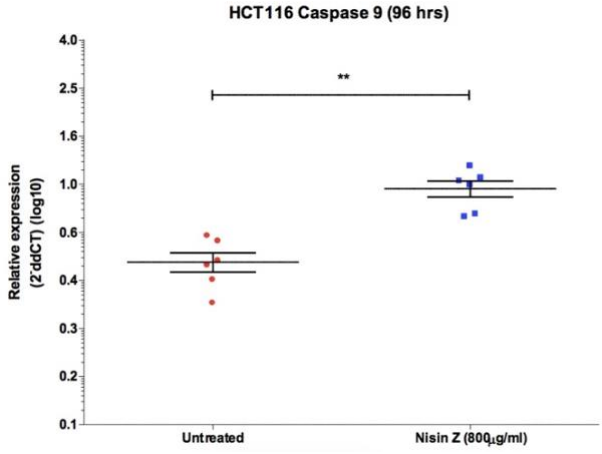
Primer name	Primer sequence
CHOP forward	5’-GGAAGTCATTGGAGGGTTTG-3’
CHOP reverse	5’-CCTCCGCAACTCTATTCACC-3’
Caspase9 forward	5’-CTGGAGTCTTAGTTGGCTACTCG-3’
Caspase9 reverse	5’-GGTCCACCTGCAGCTCTTC-3’
Caspase8 forward	5’-GGAGTGGCAGTGGTTGGA-3’
Caspase8 reverse	5’-CCAAAAACTCAGAGCACATGAC-3’
Caspase12 forward	5’-AGGAGAGCAAGCCTGTTGAA-3’
Caspase12 reverse	5’-CCGTTGGATGGTTTCTCATC-3’
BCL2 forward	5’-ACATTTGAACATGACCCATCTG-3’
BCL2 reverse	5’-AGGCCAGGCCAAATATCAC-3’
BNIP3 forward	5’-TTTTGACATTATCTTTCCTACAGCA-3’
BNIP3 reverse	5’-TGGAAAGGTGAAACATCAAGG-3’
Beclin forward	5’-CCTGCAATCACAGCCAGTT-3’
Beclin reverse	5’-GAAGGGCTCGGTCTGGAT-3’
Beta actin forward	5’-TCGTGCGTGACATTAAGGAG-3’
Beta actin reverse	5’-CAGGCAGCTCGTAGCTCTTC-3’

Table 4.2 Primer sequences used in this study for qRT-PCR. Primers designed using Universal Probe Library (Roche Life Science).

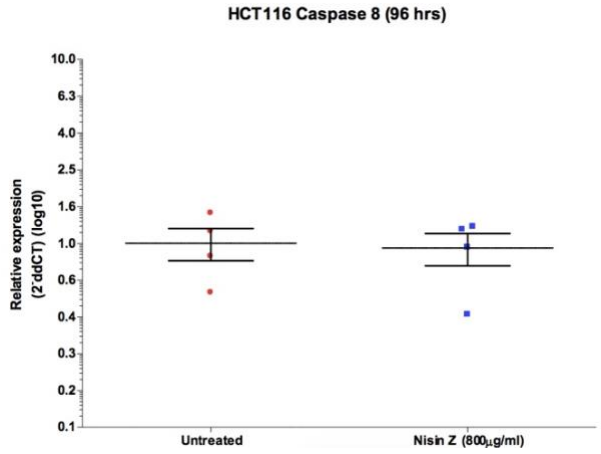
A


HCT116 CHOP (96 hrs)
Relative expression (2-ddCT) (log10)
6.3
4.0
2.5
1.6
1.0
0.6
0.4
0.3
0.2
0.1
Untreated
Nisin Z (800µg/ml)

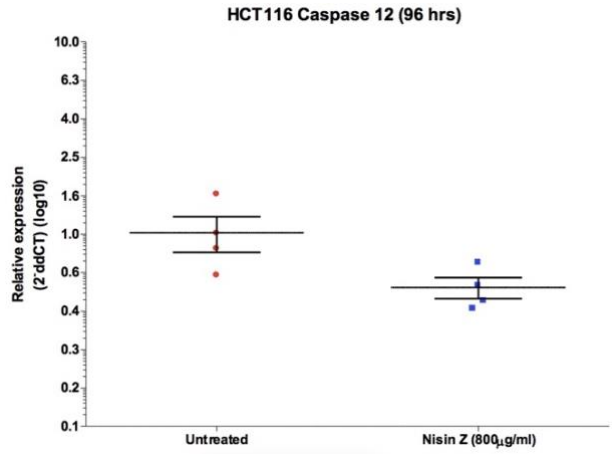
B


HCT116 Caspase 9 (96 hrs)
**
Relative expression (2-ddCT) (log10)
4.0
2.5
1.6
1.0
0.6
0.4
0.3
0.2
0.1
Untreated
Nisin Z (800µg/ml)

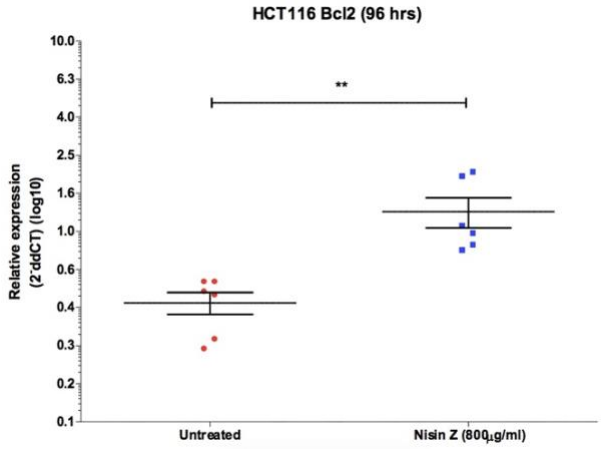
C


HCT116 Caspase 8 (96 hrs)
Relative expression (2-ddCT) (log10)
10.0
6.3
4.0
2.5
1.6
1.0
0.6
0.4
0.3
0.2
0.1
Untreated
Nisin Z (800µg/ml)

D


HCT116 Caspase 12 (96 hrs)
Relative expression (2-ddCT) (log10)
10.0
6.3
4.0
2.5
1.6
1.0
0.6
0.4
0.3
0.2
0.1
Untreated
Nisin Z (800µg/ml)

E


HCT116 Bcl2 (96 hrs)
**
Relative expression (2-ddCT) (log10)
10.0
6.3
4.0
2.5
1.6
1.0
0.6
0.4
0.3
0.2
0.1
Untreated
Nisin Z (800µg/ml)

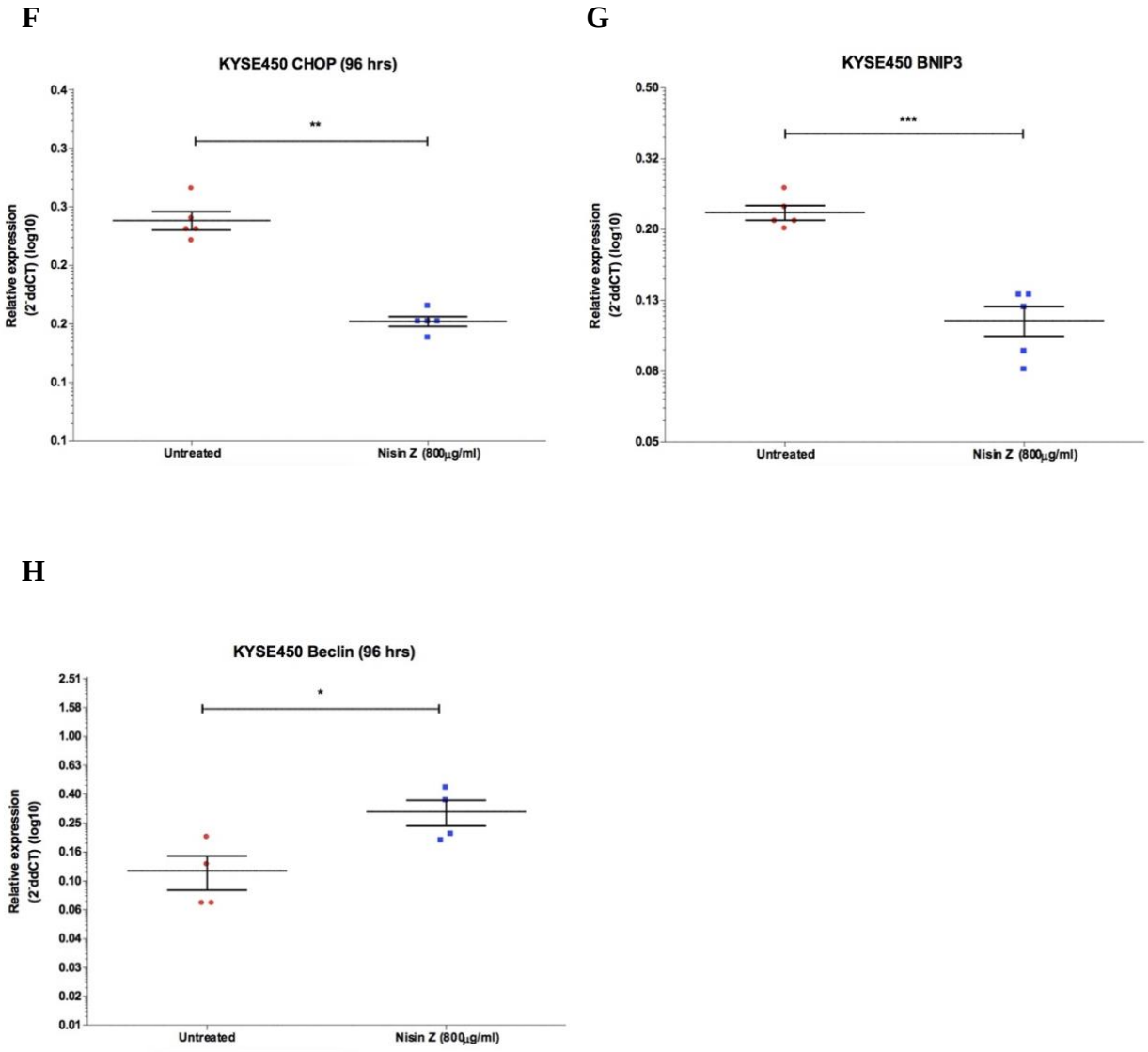


Figure 4.7 qRT-PCR results for cell lines HCT116 and KYSE450 untreated and treated with 800µg/ml nisin Z for 96hrs. In the case of HCT116 five sets of primers were evaluated: (A) CHOP, (B) Caspase 9, (C) Caspase 8, (D) Caspase 12 and (E) Bcl2. In the case of KYSE450 three sets of primers were evaluated: (F) CHOP, (G) BNIP3 and (H) Beclin.

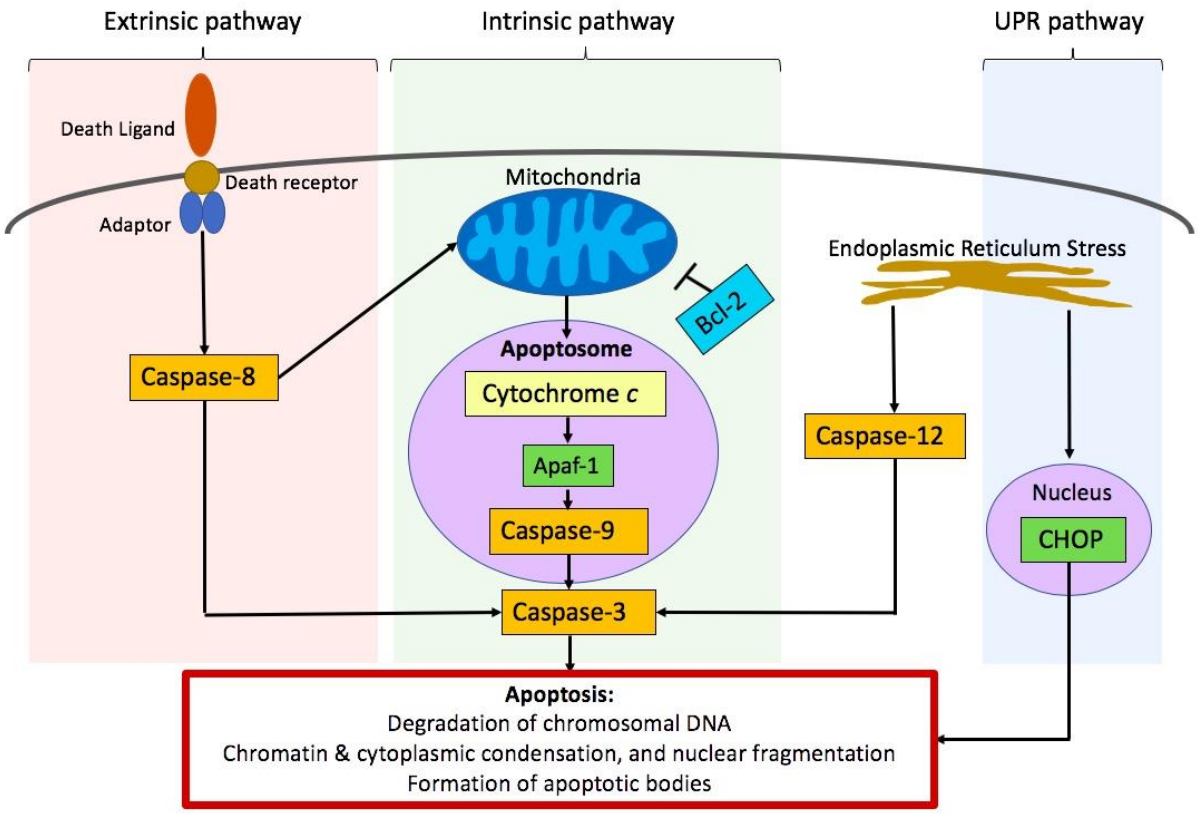


Figure 4.8 Apoptosis pathways adapted from John C. Reed and Maurizio Pellecchia, 2005, Oshitari T. et al., 2008, Susan Elmore, 2007, and S. Fulda and K-M. Debatin, 2006[27,28,31,32].

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Thesis Summary and Future Work

Lantibiotics are bacterially produced ribosomally synthesised antimicrobial peptides, distinguished by the presence of the unusual amino acids lanthionine and methyllanthionine which are responsble for the formation of bridges described in these peptides. Nisin and lacticin 3147 are both well-studied lantibiotics produced by *Lactococcus lactis*, having antimicrobial activity against a wide spectrum of microorganisms in the nanomolar range. Numerous variants of nisin have been created through bioengineering techniques to better characterise functional properties or exhibit enhanced bioactivity. Reports have also started to emerged analysing the possibility of using bacteriocins in a clinical setting. These biomedical applications of bacteriocins range from being used as alternatives to antibiotics to being adopted into cancer treatment regimes.

Chapter I of this thesis reviews the biomedical applications of bacteriocins in relation to infection and oncology. Different nisin variants are also discussed along with nisin and lacticin's synergistic effects when used in combination with other animicrobials. Furthermore nisin's innate induction system and its subsequent manipulation is reviewed.

Chapter II focuses on the nisin variant nisin-AAA's self-induction properties when amino acids at positions K12 and H31 were independently changed to every other natural amino acid. Ultimately, this study highlighted that ten nisin-AAA variants were able to self-induce without the need for an initial introduction of nisin (in the form of Nisaplin) (original nisin K12-AAA-H31, K12V, K12Q, K12W, K12T, K12A, K12C. H31N, H31K and H31R). This was apparent from deferred antagonism assays against the known sensitive indicator strain *L.lactis* HP. Further well assays showed the possible connection between induction ability and amino acid charge. Negatively charged amino acids D and E at position 12 resulted in the

decrease or loss of production and self-induction, whereas sustained self-induction was exclusive to the positively charged amino acid K, H, R (and N which is uncharged).

Chapter III describes the use of the bacteriocins, nisin Z and lacticin 3147, in combination with different antibiotics (penicillin G, vancomycin and methicillin) against different indicator strains, *C. acnes*, *E. faecium* UCC1, *E. feacium* UCC2, *C. xerosis* and MRSA ST291. Enhanced antimicrobial activity can be seen when combining lacticin 3147 with either penicillin G or vancomycin against all indicator strains. Furthermore, partial synergy between nisin Z and methicillin was apparent from FIC assays. This enhanced activity was also shown on porcine skin models *ex-vivo*. Further FIC assays should be conducted to establish specific synergistic activity between lacticin 3147 and penicillin G or vancomycin against the different indicator strains.

Chapter IV investigates the use of the bacteriocin nisin Z as a possible alternative therapeutic for colorectal and oesophageal cancers. Nisin Z was shown to induce apoptotic cell death in all colorectal cancer cell lines and possible type II (autophagic) cell death in the two oesophageal cell lines. In the case of HCT116 cell lines (colorectal) flow cytometry results showed that an intrinsic apoptotic pathway was induced by the treatment of nisin. Further analysis should be done to determine the exact cell death pathways taken by each cell line used in this study when treated with nisin Z.

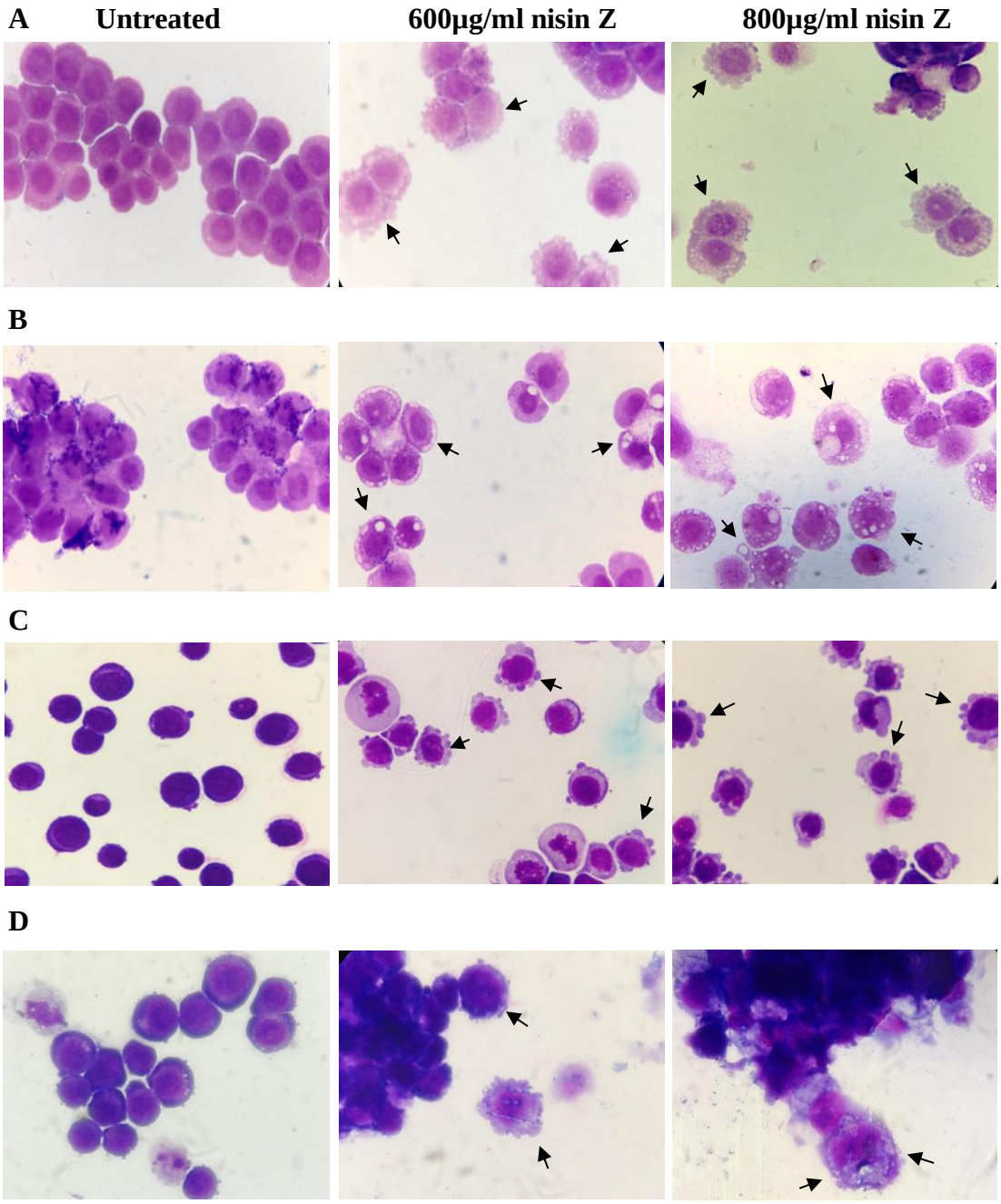
Overall, this thesis makes several contributions to our knowledge of lantibiotics and their potential biomedical applications in clinical settings. However, future work should be completed to further investigate the effect bacteriocins have on cells of the immune system. Cells of the immune system may respond variably to the presence of bacteriocins and the upregulation/downregulation of proteins such as cytokines from macrophage cells should be investigated. To date no cytotoxic effects of nisin have been reported even when tested at high doses of 83.25mg/kg of diet. However, the concentration of nisin required for therapeutic doses

well exceeds this and should be further evaluated to ensure its safety for use as a viable therapeutic.

In relation to using bacteriocins in combination with antibiotics the exact mechanism by which nisin Z and lacticin 3147 act synergistically with β-lactam antibiotics against β-lactamase producing bacteria still needs to be investigated. It may be that their different modes of action weaken the cell to an extent that it cannot recover, but it would also be prudent to determine whether β-lactamase production is down-regulated. The effect bacteriocins have on the gut microbiome should also be considered when determining their use as a therapeutic.

Eventhough bacteriocins have shown great success within the food industry and show great promise for their biomedical applications, it is clear that further research needs to be done on the pharmocokinetic and phamacodynamic properties of administering high therapeutic doses of bacteriocins before admiting them to be used as biological therapeutics.

Appendix



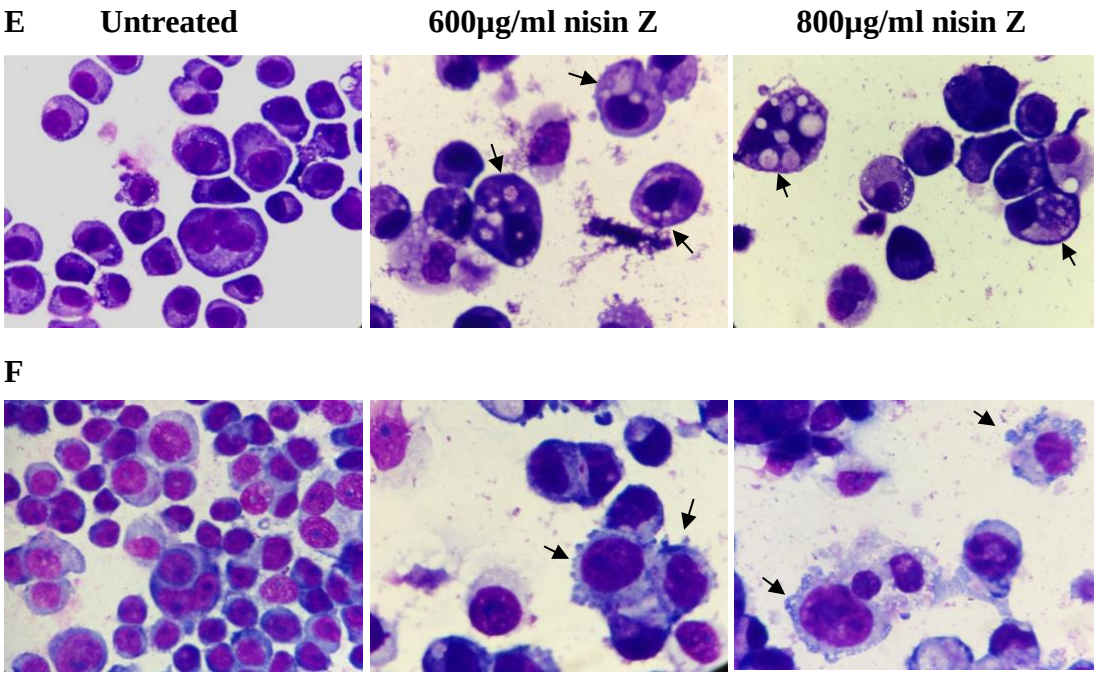


Figure 5.1 Cytospin results of: (A) HT29, (B) OE19, (C) SW620 and (D) Caco2 cell lines 72 hrs post treatment, and (E) KYSE450 and (F) HCT116 cell lines 96 hrs post treatment. Figures represent untreated and treated cell with 600 µg/ml nisin Z and 800 µg/ml nisin Z for all cell lines. In the cases of (A), (C), (D) and (F) arrows indicate cytoplasmic blebbing, and in the case of (B) and (E) arrows indicate autophagic vacuoles.

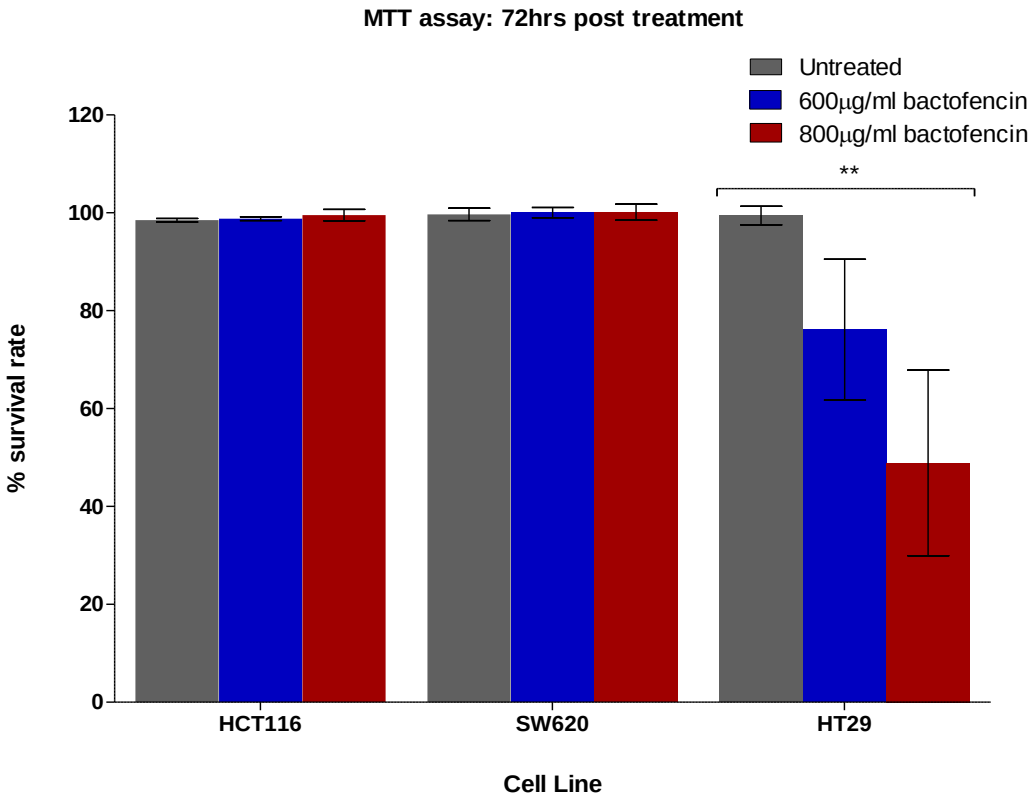


Figure 5.2 MTT results % survival results of cell lines HCT116, SW620 and HT29 96 hrs post treatment with 400 µg/ml, 600 µg/ml and 800 µg/ml of bactofencin A.

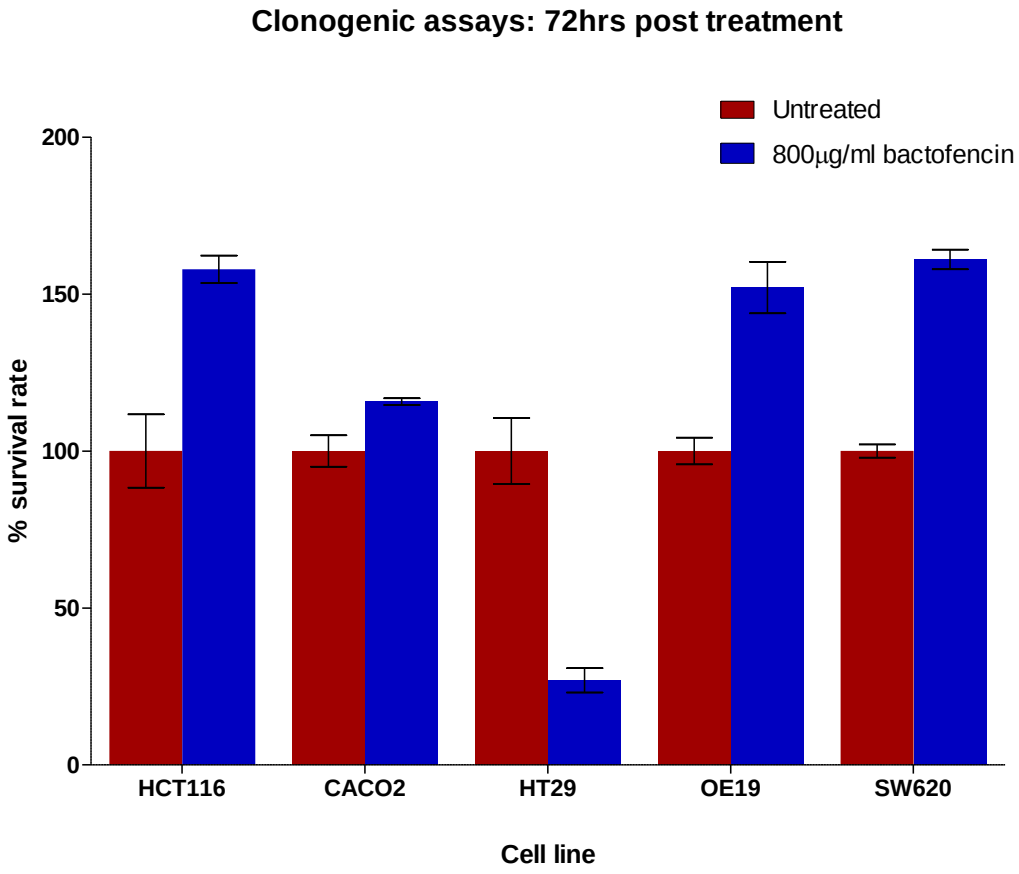


Figure 5.3 Clonogenic results of cell lines HCT116, Caco2, HT29, OE19 and SW620 untreated and treated with 800 µg/ml of bactofencin A for 72 hrs.