Zoocin A and lauricidin in combination selectively inhibit *Streptococcus mutans* in a biofilm model

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A thesis submitted for the degree of
Doctor of Philosophy
at the University of Otago, Dunedin,
New Zealand.

November 2010
Abstract

The oral cavity contains many different microbial species growing in a biofilm. Dental caries is the localised destruction of the tooth by organic acids produced from the bacterial fermentation of dietary carbohydrates. The mutans streptococci, in particular *Streptococcus mutans*, have been proposed as the main etiological agents of dental caries and high levels of mutans streptococci in the plaque is correlated with a higher risk for dental caries. A range of broad-spectrum antimicrobials are used to inhibit plaque formation. However, current research is focussed on more targeted approaches. The protein zoocin A has high activity against *Streptococcus mutans*, while the monoglyceride lauricidin is active against gram-positive bacteria. Both are therefore potential antimicrobials for anti-caries therapy. The aims of this study were to produce zoocin A at sufficient concentrations to use as an antimicrobial in a biofilm model and to develop a simple biofilm model for use studying the effects of zoocin A and lauricidin upon an oral biofilm.

Zoocin A was produced as a recombinant protein in *Escherichia coli*. Zoocin A production of 165 mg per litre of culture was achieved by the use of a benchtop fermentor. Neither optimisation of codon usage or expression of zoocin A in *E. coli* BL21 CodonPlus-(DE3)-RIL resulted in increased yields of zoocin A.

A triple-species biofilm model was developed where *S. mutans*, *Streptococcus oralis* and *Actinomyces viscosus* were grown on glass beads and supplied 1/3 strength brain heart infusion (BHI) supplemented with 1% sucrose as a nutrient source. Zoocin A and lauricidin were added directly to the feed medium, both individually and in combination. Biofilms were incubated for 24, 48 or 72 hours, then each species was enumerated and biofilm formation and the pH were measured. The addition of 40 µg/ml zoocin A to the biofilm model specifically reduced the levels of *S. mutans* in the biofilm by approximately two logs and was accompanied by an increase in the levels of *S. oralis*. Lauricidin reduced *S. mutans* by a similar amount when applied at 10 µg/ml. The use of zoocin A and lauricidin in combination resulted in a four log decrease in the level of *S. mutans*, while levels of *S. oralis* and *A. viscosus* remained elevated. Zoocin A and lauricidin also reduced biofilm formation and maintained the pH above 7.0, while the pH of control biofilms decreased to 4.3 after 72 hours. The effects of chlorhexidine on biofilm formation and pH were similar. However, *S. mutans* was reduced by approximately five logs and a reduction in the colony forming units of...
all three species was seen. The application of zoocin A and lauricidin to the biofilm model gave a targeted reduction in *S. mutans*. While the reduction was not as large as that seen with chlorhexidine, zoocin A and lauricidin did not reduce other bacterial species in the biofilm and gave a similar reduction in biofilm formation and maintenance of pH. There was little or no development of resistance to zoocin A or lauricidin by biofilm cells.

This study suggests that antimicrobial agents can be used in combination in the oral cavity to achieve a targeted inhibition of *S. mutans*. Further research is required to optimise the dosage and the best route of delivery.
Acknowledgements

First and foremost, thanks to my supervisor Dr Robin Simmonds, who has supported me throughout my studies. His patient discussions and assistance have been invaluable.

My grateful thanks also go to my supervisory panel: Professor John Tagg, Associate Professor Margaret Baird, Dr Heather Brooks and Dr Geoffrey Tompkins, for their helpful conversations and encouragement.

And thank you to everyone else who has supported me and assisted with this project, in particular:

Dr Muriel DuFour, for assisting me in the laboratory with new methods and techniques.

Liz Girvan, (Microscopy Otago, University of Otago) for the preparation of my electron microscopy samples and her assistance with the use of the scanning electron microscope.

Members of the Department of Microbiology and Immunology, who have given me advice, encouragement or support during my studies.

Past and present members of the Simmonds and Brooks labs, for their encouragement, discussion and for making bench work more fun.

James, Anthony, Rebekah, Jocelyn and previous members of the Thursday lunch crowd, for providing a refreshing break from laboratory work and moral support during difficult times.

Felicity and Anthea, for their friendship and for always being ready with cake – to celebrate when experiments worked or to commiserate when things went wrong.

My parents, Chris and Karen Hedges, for their constant encouragement and enthusiasm, and for being so fantastic.

And my husband, Hayden, for his love, encouragement and unfailing support during my studies. Thank you.
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulphate</td>
</tr>
<tr>
<td>AU</td>
<td>arbitrary units</td>
</tr>
<tr>
<td>BA</td>
<td>blood agar</td>
</tr>
<tr>
<td>BDH</td>
<td>British Drug Houses Chemicals Ltd</td>
</tr>
<tr>
<td>BHI</td>
<td>brain heart infusion broth</td>
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<tr>
<td>BLIS</td>
<td>bacteriocin-like inhibitory substance</td>
</tr>
<tr>
<td>BMM</td>
<td>basal medium mucin</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAB</td>
<td>columbia blood agar base</td>
</tr>
<tr>
<td>CAB + rif</td>
<td>CAB agar with 100 μg/ml rifampicin</td>
</tr>
<tr>
<td>CAB + strep</td>
<td>CAB agar with 100 μg/ml streptomycin</td>
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<tr>
<td>CDFF</td>
<td>constant depth film fermentor</td>
</tr>
<tr>
<td>CFAT agar</td>
<td>cadmium fluoride acriflavine tellurite agar</td>
</tr>
<tr>
<td>cfu</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>DLS</td>
<td>dye labelled substrate</td>
</tr>
<tr>
<td>DRA</td>
<td>dye release assay</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EPS</td>
<td>extracellular polysaccharide</td>
</tr>
<tr>
<td>FIC</td>
<td>fractional inhibitory concentration</td>
</tr>
<tr>
<td>FITC-WGA</td>
<td>fluorescein isothiocyanate conjugated wheat germ agglutinin</td>
</tr>
<tr>
<td>GRAS</td>
<td>generally regarded as safe</td>
</tr>
<tr>
<td>GTF(s)</td>
<td>glucosyltransferase(s)</td>
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<tr>
<td>His-tag</td>
<td>polyhistidine-tag</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
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<tr>
<td>ID</td>
<td>internal diameter</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-thio-β-D-galactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
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<tr>
<td>LBA</td>
<td>Luria broth agar</td>
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<tr>
<td>LBA + amp</td>
<td>LBA with 100 μg/ml ampicillin</td>
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<tr>
<td>LBA + amp + kan</td>
<td>LBA with 100 μg/ml ampicillin and 25 μg/ml kanamycin</td>
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<td>LBA with 34 μg/ml chloramphenicol</td>
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<td>LBA with 25 μg/ml kanamycin</td>
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<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>MQ water</td>
<td>milli-Q deionised water</td>
</tr>
<tr>
<td>NBS</td>
<td>New Brunswick Scientific</td>
</tr>
<tr>
<td>NC</td>
<td>no change</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OD&lt;sub&gt;595&lt;/sub&gt;</td>
<td>optical density at 595 nm</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
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<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
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<tr>
<td>RO water</td>
<td>reverse osmosis water</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
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<tr>
<td>SAG</td>
<td>salivary agglutinin glycoprotein</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SLS</td>
<td>sodium lauryl sulphate</td>
</tr>
<tr>
<td>SOB</td>
<td>super optimal broth</td>
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<tr>
<td>SOC</td>
<td>SOB with catabolite repression</td>
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<tr>
<td>TAE buffer</td>
<td>tris-acetate ethylenediaminetetraacetic acid buffer</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N′,N′-tetramethylethylene diamine</td>
</tr>
<tr>
<td>THB</td>
<td>Todd Hewitt broth</td>
</tr>
<tr>
<td>T&lt;sub&gt;m&lt;/sub&gt;</td>
<td>melting temperature</td>
</tr>
<tr>
<td>TSB</td>
<td>tryptic soy broth</td>
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1. Introduction

1.1 Introduction

The oral cavity contains many different bacterial species which form a complex biofilm community. Dental caries is the localised destruction of the tooth by organic acids produced by the bacterial fermentation of dietary carbohydrates, particularly sucrose. Dental caries is an infectious disease,\textsuperscript{216,452} which is linked to the presence of the mutans streptococci.\textsuperscript{54,372,445} The mutans streptococci are particularly acidogenic and aciduric,\textsuperscript{310,445} and therefore cause a reduction of plaque pH when carbohydrates are ingested.\textsuperscript{448,452} Individuals with the highest numbers of mutans streptococci in their oral cavity are at greatest risk of developing dental caries\textsuperscript{275} and therefore suppression of this population is thought to reduce that risk. Currently prevention of dental caries aims to prevent dental plaque build up or to reduce the levels of mutans streptococci in the plaque.\textsuperscript{28,415} Several chemical agents currently used in dental products suppress the growth of mutans streptococci.\textsuperscript{41,122,344,395} Other possible prevention options include the use of novel antimicrobial compounds,\textsuperscript{101,235,269} using artificial sweeteners instead of sucrose in the diet,\textsuperscript{439} replacement therapy of cariogenic bacteria with avirulent strains,\textsuperscript{427} and anti-mutans vaccination.\textsuperscript{401} Of these possible options, most attention has focussed on the use of small antibacterial compounds to inhibit plaque formation, and the development of new antimicrobial therapies remains a priority. The protein zoocin A has high activity against \textit{S. mutans},\textsuperscript{391} making it a candidate for anti-caries therapy along with lauricidin, a broad-spectrum antimicrobial.\textsuperscript{74,376} To use a protein such as zoocin A as an anti-caries agent it must be produced in large quantities. Recombinant \textit{E. coli} is often used for this purpose and the protein can then be purified by the use of affinity tags.\textsuperscript{251,433} To test the effectiveness of anti-cariogenic antimicrobials, initially plaque models are utilised. These may include pure or co-cultures, which are often used in preliminary studies on an antimicrobial’s activity due to their simplicity.\textsuperscript{291,409} Biofilm models are more complex, but they are able to more accurately model the oral cavity and allow investigation into the effects of bacterial interactions.\textsuperscript{428} Biofilm models are also important as the effectiveness of antimicrobials is often reduced in biofilms.\textsuperscript{14,15} Animal models allow more detailed investigation into bacterial pathogenesis and disease treatment,\textsuperscript{465} however, ultimately human trials must be undertaken to determine the efficacy of any new antimicrobial.\textsuperscript{87,342}
1.2 Oral bacteria and biofilms

Over 700 bacterial species have been detected in the oral cavity and a study using pyrosequencing estimated the number of phylotypes to be more than 19,000. In an individual the number of bacterial species present is much lower, but these bacteria form a complex biofilm. The oral biofilm is a three dimensional, structured community of bacteria attached to the oral surfaces, notably tooth enamel. Oral biofilm development occurs in stages: initial attachment of the early colonising bacteria; maturation of the biofilm, as secondary colonisers attach; and dispersal, as organisms are lost from the mature biofilm.

The initial stages of oral biofilm development involve the acquired pellicle. This forms immediately after tooth cleaning, when a film of saliva coats the tooth surface. Host derived molecules in the saliva form the acquired pellicle, which contains various enzymes, mucins, glycoproteins, proline-rich proteins, phosphate-rich proteins and sialic acids. These compounds in the acquired pellicle act as receptors to which the early colonising bacteria attach. The early colonisers are predominantly streptococci, particularly Streptococcus mitis and S. oralis, along with Streptococcus gordonii and Streptococcus sanguinis. The Actinomyces species are also significant early colonisers and other primary colonisers include bacterial species such as those of Eikenella, Neisseria, Haemophilus, Prevotella, Propionibacterium and Veillonella. These early colonisers initially adhere with specific surface adhesins, then become irreversibly attached to the pellicle components. The specific adhesins and receptors also lead to cell-to-cell recognition and adhesion between specific bacterial species, called coaggregation. This coaggregation mediates the further development of the biofilm. Streptococci demonstrate extensive coaggregation, particularly intra-species coaggregation, which is not seen with other oral bacteria. This leads to the formation of an interconnected network of early colonisers attached to the acquired pellicle and each other. Secondary colonisers then attach to this network, also through coaggregation. A key secondary coloniser is Fusobacterium nucleatum, which coaggregates with many late colonising bacteria and acts as a bridge between the early and late colonisers. Late colonisers include Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola, Actinobacillus actinomycetemcomitans, Prevotella intermedia, Selenomonas flueggei and Veillonella, Corynebacterium, Fusobacterium, Prevotella and Eubacterium species.
The bacterial cells in the biofilm are surrounded by a complex matrix which includes: secreted polymers, absorbed nutrients, metabolites, products from cell lysis, and particulate material from the surrounding environment. The extracellular polysaccharide (EPS) in this matrix primarily consists of water soluble and insoluble glucans and fructans produced by S. mutans, and promotes adhesion and biofilm formation. The process of biofilm formation results in an organised structure where primary colonisers are attached to the acquired pellicle and each other, and the secondary colonisers are attached to the microbial complex formed by the primary colonisers. Mature biofilms take several days to develop and have been shown to have a structured and heterogeneous architecture, consisting of a thin basal layer on the acquired pellicle, with multibacterial columns extending away from the substratum. These large bacterial aggregates are separated by fluid-filled pores, channels and spaces, and on the outer layers of the biofilm shear forces act to remove bacteria from the biofilm.

1.3 Bacteria and dental caries

Several hypotheses regarding the formation of dental caries have been proposed. The specific plaque hypothesis suggests that of the numerous bacterial species inhabiting the mouth only a few are actively involved in the disease, while the non-specific plaque hypothesis proposes that dental caries is the result of the combined activity of the total oral microflora. These two theories have been somewhat combined in the more recent ecological plaque hypothesis, which says organisms associated with disease may be present in non-diseased locations but at levels too low to invoke a clinical manifestation of the disease. Disease is therefore due to a shift in the balance of the resident microflora to a cariogenic state in response to a change in local environmental conditions.

The mutans streptococci (including S. mutans and Streptococcus sobrinus) have been most frequently implicated in the development of dental caries. However, dental caries lesions contain a diverse range of bacterial species, most of which have not been cultivated, and the involvement of individual bacterial species’ in the initiation of dental caries is still being established. Many other bacteria including other streptococci, such as S. mitis, Streptococcus salivarius and Streptococcus anginosus, along with Enterococcus faecalis, A. viscosus, Actinomyces naeslundii and some lactobacilli, are also thought to influence caries development.
Becker et al.\textsuperscript{38} used a reverse checkerboard assay to examine the association of 23 different bacterial species with dental caries. Nine species were found to be associated with caries: \textit{Actinomyces gerencseriae}, \textit{Bifidobacterium}, \textit{S. mutans}, \textit{Veillonella}, \textit{Streptococcus salivarius}, \textit{Streptococcus constellatus}, \textit{Streptococcus parasanguinis} and \textit{Lactobacillus fermentum}. \textit{A. gerencseriae}, \textit{S. mutans} and \textit{S. salivarius} were the only species to be significantly associated with early caries lesions (white spots). This suggests these bacteria may be associated with the initiation of dental caries, while other caries associated bacteria, notably \textit{Bifidobacterium}, are more likely to be involved in the progression of caries lesions. Aas et al.\textsuperscript{1} used 16S RNA sequencing to identify species present in caries lesions and from sound tooth surfaces of individuals both with and without caries. Ten percent of the individuals with rampant caries did not have detectable levels of \textit{S. mutans}, indicating other oral bacteria are involved in caries development. However, \textit{S. mutans} was still significantly associated with caries lesions. van Houte et al.\textsuperscript{448} found that non-mutans streptococci isolated from caries lesions were more acidogenic than non-mutans streptococci isolated from sound tooth surfaces and that the non-mutans streptococci are heterogeneous for acid production. The authors suggested that cariogenic conditions were associated with increased proportions of acidogenic and aciduric bacteria and that a shift to these conditions involves bacterial species other than the mutans streptococci. Beighton\textsuperscript{40} has suggested that while mutans streptococci are good markers of disease, they are not necessarily the etiological agents of dental caries. However, despite evidence that a range of bacteria are involved in dental caries, the presence of mutans streptococci is a key risk factor for the development of caries\textsuperscript{372} and consideration of these risk factors can reduce caries incidence.\textsuperscript{380}

The interactions within this complex community are still being unravelled and interactions between cariogenic and non-cariogenic bacteria can change the cariogenic potential of a plaque sample.\textsuperscript{225} Wang and Kuramitsu\textsuperscript{463} found that the growth of \textit{S. mutans} in the presence of \textit{S. gordonii} inhibited \textit{S. mutans} bacteriocin production. \textit{S. gordonii} produced a protease that degraded the competence-stimulating peptide, a quorum sensing regulator of \textit{S. mutans} required for bacteriocin production. Several studies have also shown \textit{S. gordonii} and \textit{Streptococcus sanguis} reduce biofilm formation by \textit{S. mutans};\textsuperscript{241,245,472} however, when \textit{S. mutans} was grown with \textit{Lactobacillus casei}, biofilm formation increased but the expression of genes linked to some virulence factors was reduced.\textsuperscript{472}
1.3.1 Cariogenic bacteria

There are several properties that are considered distinctive of cariogenic bacteria. They must be able to rapidly transport sugars when in competition with other plaque bacteria, convert these sugars rapidly into acid and have the ability to maintain these activities at the low pH that results from the metabolism of the sugars.354,447,449

*S. mutans* is able to metabolise a wide range of carbohydrate. Genome analysis has found genes for the transport and metabolism of glucose, fructose, sucrose, lactose, galactose, mannose, cellobiose, β-glucosides, trehalose, maltose, raffinose, ribulose, mellobiose, starch and isomaltosaccharides.7 The fermentation of these carbohydrates provides energy for the cell and results in acid production. Mutans streptococci are heterofermentative, allowing them to produce mixed-acid end-products, giving them a growth advantage over other plaque bacteria.393 They also store non-metabolised carbohydrate in the form of intracellular polysaccharides447 and when the external source is limited they can continue growth and metabolism by utilising this stored carbohydrate.393 This leads to the extended production of acid following carbohydrate ingestion and thus a lowering of the dental plaque pH.268,452 Sucrose is particularly important in dental caries. Dental caries has been linked to the frequency of sucrose ingestion139,444 and the presence of sucrose is essential for biofilm formation, an important virulence factor for *S. mutans* (see section 1.3.2). Sucrose is also easily metabolised by plaque bacteria to acid and the lowered plaque pH predisposes the enamel to caries formation.354,444 Following exposure to sugar under dietary conditions, high concentrations of lactic acid rapidly build up in human dental plaque and the pH decreases.139,449 Increases in both the frequency and length of sugar exposure prolong the period of low pH in the plaque.139 This lowering of the pH caused by acid production leads to caries development by demineralisation of the teeth.268,414,448 The pH at which teeth begin to demineralise varies, as it is based on ion concentrations in the solution and tooth, but is in the range of 5.3 to 5.7.98

Cariogenic bacteria must be able to survive at the low pH which results from carbohydrate fermentation. Van Houte et al.448 found bacteria isolated from human caries lesions were more acid tolerant than those isolated from sound tooth surfaces. The bacteria isolated from these caries lesions were mostly mutans streptococci. The non-mutans streptococci isolated from the lesions were more acid tolerant than the non-mutans streptococci isolated from sound surfaces.448 Stephan414 compared the oral pH of caries-active and caries-free
individuals after a glucose rinse and found those with active caries had a lower oral pH and it remained low for a longer period of time than the oral pH of the caries-free individuals. While most oral bacteria cannot grow when the pH is below five, some can maintain glycolytic processes when the pH is as low as four. Glycolytic enzymes are very sensitive to acid conditions, however mutans streptococci maintain their internal pH at neutral levels. As the bacterial membrane is not impermeable to protons, a proton-translocating F-ATPase works to move the protons out of the cell and maintain the internal pH. This allows the continuation of glycolysis at low pH. The continuation of glycolysis provides the energy to maintain the F-ATPase and thus the internal pH. Therefore the presence of sucrose has been shown to enhance the aciduricity of S. mutans as it provides a substrate for glycolysis. This mechanism can protect S. mutans from acid killing at pH levels as low as 2.5.

1.3.2 Biofilm formation by Streptococcus mutans

Biofilm formation is an important virulence factor for S. mutans. The bacteria must be able to adhere to be maintained in the oral cavity. Giacaman et al. found there was no association between the levels of mutans streptococci in the saliva and caries status but there was an association between the amount of biofilm-forming mutans streptococci and caries incidence. When cells are growing in a biofilm bacterial susceptibility to antimicrobials is often reduced and in established biofilms the antimicrobial effect is limited to the superficial layers. Sucrose is extremely important in S. mutans biofilm formation. The presence of sucrose decreases the antimicrobial susceptibility of S. mutans, probably due to the subsequent biofilm formation. Initially, or in the absence of sucrose, S. mutans adheses using ionic and lectin-like interactions. Streptococcal protein antigen P binds to salivary agglutinin glycoprotein (SAG) and SAG coated surfaces. In the presence of sucrose, glucans are synthesised by glucosyltransferases (GTFs). They form an extracellular layer of EPS that promote adhesion and biofilm formation. S. mutans produces at least three GTFs encoded by the genes gtfBCD. The product of gtfB produces water-insoluble glucans, the gtfC encoded enzyme produces both soluble and insoluble glucans, while the product of gtfD produces water-soluble glucans. All three GTFs are required for optimum biofilm formation, however glucosyltransferase C, the product of gtfC, appears to be particularly important. Inactivation of the GTFs reduced the virulence of S. mutans in a rat model, specifically S. mutans lost the ability to produce water insoluble glucans and had reduced
surface adherence. Glucan binding proteins produced by *S. mutans* also promote cell aggregation and biofilm formation and the loss of glucan binding proteins can affect biofilm formation and structure.

### 1.4 Preventing dental caries

The incidence of dental caries can be reduced by efficient oral hygiene which involves the mechanical removal of plaque, particularly when this is combined with a reduction of sugar in the diet. However public compliance is low and treatment of the carious lesion usually involves the surgical removal of the diseased part of the tooth. Investigation into preventative measures which either reduce plaque or prevent caries lesions forming is therefore highly desirable.

#### 1.4.1 Chemical agents

**1.4.1.1 Fluoride**

The administration of fluoride systemically significantly reduces caries incidence. Fluoride affects plaque bacteria by a range of mechanisms. It inhibits the transport of sugars into the cell and reduces the activity of phosphopyruvate dehydratase, an enzyme involved in glycolysis. Fluoride also inhibits the F-ATPase, which is essential for moving hydrogen ions out of the cell to maintain the internal pH. Since this process requires energy, which, in low pH environments, is provided by glycolysis, the F-ATPase is also affected by the fluoride inhibition of sugar transport and glycolysis. The inhibition of glycolysis results in reduced substrate for the formation of extracellular glucans and fluoride also inhibits the formation of water-insoluble glucans, without affecting the formation of water-soluble sugars. Fluoride is taken into the cell as hydrogen fluoride and is transported more rapidly in low pH environments, when the difference between the internal and external pH is greater. As the internal pH is more alkaline than the external pH, the hydrogen fluoride then dissociates. Fluoride therefore increases the uptake of protons, again reducing the bacterial ability to survive in acidic environments. Thus bacteria are more susceptible to fluoride in low pH environments. Fluoride inhibits acid phosphatase, which is involved in the release of phosphate from the enamel during demineralisation and induces a release of phosphate from cells. The combination of these two effects is thought to promote remineralisation. Shani et al. found amine
fluorides also have antimicrobial properties, associated with their cationic properties. Amine fluorides also inhibit glucan production and reduce caries incidence.

There is some evidence that fluoride reduces \textit{S. mutans} adhesion. Studies have shown the fluoride ion is bactericidal against oral streptococci \textit{in vitro}, particularly against \textit{S. mutans}, while \textit{in vivo} it has been suggested that fluoride is unable to modify the plaque composition when added to the diet or water supply. However, topical applications of high concentrations of fluoride reduced the levels of \textit{S. mutans} in the plaque and fluoride on the substratum (hydroxyapatite) inhibited biofilm growth of \textit{S. mutans}, when glucose was in excess and the pH below five. Although low levels of fluoride were not bacteriocidal \textit{in vivo}, fluoride at 1 mM reduced the population change and pH decrease seen in the plaque following a glucose challenge and prevented the enrichment of mutans streptococci in the plaque. Therefore sub-inhibitory concentrations of fluoride in the saliva may provide an anti-caries effect. Studies have found the use of fluoride containing products elevates levels of fluoride in the saliva and plaque. Campus et al. found regular use of fluoride containing products (three times a day) maintained salivary fluoride at clinically useful concentrations and that the concentration was maintained 24 hours after product use was halted. Zero et al. found fluoride is also absorbed into the oral soft tissues, which act as an intra-oral reservoir, releasing fluoride over time. However, despite the anti-caries effects of fluoride, it cannot entirely prevent demineralisation and changes in the biofilm matrix when sucrose challenge is frequent.

\textbf{1.4.1.2 Chlorhexidine}

The most studied, and most effective antimicrobial agent in use is chlorhexidine, a cationic bisbiguanide. Chlorhexidine is a broad-spectrum antimicrobial, active against a range of gram-positive and gram-negative bacteria such as \textit{A. viscosus}, \textit{Staphylococcus aureus}, \textit{Staphylococcus epidermidis}, \textit{Pseudomonas aeruginosa}, \textit{Micrococcus luteus}, \textit{E. coli} and \textit{Proteus}, \textit{Klebsiella}, \textit{Propionibacterium} and \textit{Selenomonas} species, as well as a range of streptococci including \textit{S. mutans}, \textit{S. sanguis}, \textit{S. mitis}, \textit{S. salivarius} and \textit{E. faecalis}. Stanley et al. tested a range of species isolated from patients with chronic inflammatory periodontal disease for sensitivity to chlorhexidine. All the species tested, including some streptococci, actinomyces, lactobacilli and \textit{Bacteroides} species, were sensitive to chlorhexidine.

Chlorhexidine acts by disrupting the bacterial cell membrane, affecting its permeability. The cationic molecule is thought to rapidly bind to the negatively charged cell which
increases the permeability of the cell membrane. Meurman et al. found concentrations of 0.1% chlorhexidine caused cell wall disruption, vacuolisation, sloughing and leakage of the cytoplasm and reduced cellular adherence in \textit{S. mutans}. At low concentrations chlorhexidine is bacteriostatic and at higher concentrations it becomes bacteriocidal. It is thought to bind to bacteria, salivary proteins and the tooth surface and is maintained in the oral cavity for at least 24 hours following use in a mouthwash. The concentrations remaining in the oral cavity are low, however these concentrations are bacteriostatic for some species, and lower concentrations of chlorhexidine may prevent plaque formation by preventing the formation of the salivary pellicle, preventing attachment of bacteria to the pellicle and reducing bacterial agglutination in the plaque. Mutans streptococci are particularly sensitive to the effects of chlorhexidine and it causes cell lysis of \textit{S. mutans} and reduced viability in surviving cells. Chlorhexidine also inhibits the growth of mixed bacterial populations and mixed species biofilms.

Chlorhexidine has several undesirable side effects with frequent use. The most common are a brown staining of the teeth and tongue, especially when used in combination with some dietary factors such as coffee and tea, and altered taste perception, particularly for salty flavours. Occasionally increased calculus formation, mucosal desquamation and tooth sensitisation can occur.

\subsection{1.4.1.3 Other chemical compounds}

Other chemicals have also been shown to have an antimicrobial effect. The surface acting compound delmopinol reduces plaque formation and reduces the acid production of several oral streptococci strains. Delmopinol also reduces glucan synthesis. Rundegren et al. found that biofilms treated with 0.2% delmopinol reduced glucan synthesis and biofilm adherence, making the cells more susceptible to mechanical removal. Simonsson et al. found delmopinol removed established plaque \textit{in vitro} in an \textit{S. mutans} biofilm model. However, delmopinol is less effective than chlorhexidine. When tested against a range of oral bacteria, including streptococci, actinomycetes and lactobacilli, delmopinol had a minimum inhibitory concentration (MIC) 5 to 125 times higher than chlorhexidine. Short and long term clinical studies have shown delmopinol can reduce plaque formation. Lang et al. found a 0.2% delmopinol hydrochloride rinse reduced plaque formation when used twice a day. It was less effective than a 0.2% chlorhexidine rinse, but more acceptable to the patients. Tooth staining and taste perception changes occurred with both mouthwashes but were less severe with the mouthwashes containing delmopinol.
The food preservatives sodium benzoate, potassium sorbate and sodium nitrite reduce *S. mutans* biofilm formation\(^9\) and zinc salts reduce acid production by oral streptococci.\(^{162}\) Zinc has also been used in combination with triclosan.\(^{56}\) Triclosan inhibits the F-ATPase, involved in acid tolerance of *S. mutans*, and increases membrane permeability.\(^{344}\) It also inhibits glycolytic enzymes and transport of sugars into the cell.\(^{344}\) Triclosan acts additively with fluoride\(^{344}\) and zinc citrate. Brading et al.\(^{56}\) found a zinc citrate and triclosan formulation was more effective at inhibiting glycolysis than triclosan alone and in clinical trials triclosan\(^{192,358}\) and triclosan in combination with zinc citrate\(^{199,416,424}\) reduced plaque formation. Triclosan also reduced the total bacterial count in an *in vitro* biofilm model\(^{148}\) and in combination with an amine-fluoride, reduced plaque formation in a clinical trial.\(^{20}\)

### 1.4.2 Other prevention options

Artificial sweeteners promote saliva flow without causing acid production. They can have weak antimicrobial effects, they are not metabolised by most plaque bacteria and are metabolised only slowly by mutans streptococci.\(^{439}\) Xylitol is a common artificial sweetener. It is antimicrobial to *S. mutans* at low concentrations\(^{405,443}\) but does not kill lactobacilli, actinomyces or most other streptococcal species.\(^{443}\) As xylitol is not easily metabolised by plaque bacteria,\(^{160}\) it can reduce the acid production caused by sugar metabolism when used in place of simple sugars.\(^{112}\) In clinical trials xylitol reduced the levels of *S. mutans* in the saliva\(^{308}\) and plaque.\(^{156,288}\) Xylitol exposure reduced the proportion of soluble glucans produced by *S. mutans in vitro*\(^{404}\) and in human plaque.\(^{289}\) Isokangas et al.\(^{185}\) found *S. mutans* colonisation of children could be reduced by regular use of xylitol by the mother during the child’s first two years of life.

Vaccination has also been suggested as a method to prevent dental caries. Specific immunity to oral bacteria can be provided by secretory immunoglobulin (Ig) A in the saliva.\(^{306}\) Passive immunisation by topical application of anti-mutans monoclonal antibody to the teeth of human subjects led to a significant delay in recolonisation.\(^{283}\) Kruger et al.\(^{243}\) developed a recombinant lactobacilli strain which constitutively expressed a single chain antibody against a *S. mutans* surface antigen. When introduced to a rat caries model the recombinant strain was detected in the oral cavity throughout the study and reduced the incidence of dental caries. Vaccination of rats with recombinant phosphopyruvate dehydratase from *S. sobrinus* resulted in specific IgA production and decreased dental caries in the immunised rats.\(^{109}\)
reduction of dental caries frequency also occurred in rats vaccinated with an intranasal prime-boost immunisation consisting of a DNA prime (encoding the surface antigen PAc) followed by a boost of recombinant PAc protein. The prime-boost strategy induced a stronger immune response than either component individually. Childers et al. found human oral immunisation with antigen-containing liposomes induced a salivary IgA response specific to \textit{S. mutans} glucosyltransferases. The IgA produced was primarily IgA, which is advantageous, as it is resistant to IgA proteases produced by many oral bacteria. However, human vaccination for dental caries still has many challenges concerning the selection of antigens that avoid the development of cross-reacting antibodies and the method of vaccine delivery.

Replacement therapy attempts to replace the cariogenic bacteria present in the plaque with a more benign species. However the high intrinsic stability of indigenous microflora makes this difficult and the replacement strain must therefore be highly competitive. Hillman et al. suggested an avirulent strain of mutans streptococci was more likely to occupy the same ecological niche as the cariogenic bacteria and therefore be more competitive. A strain of \textit{S. mutans} which produced high levels of mutacin, a lantibiotic which inhibits most other mutans streptococci, was found to persist in the oral cavity of three human subjects for at least 52 weeks. This strain could still be isolated in follow-up studies 3 and 14 years later. This strain was used to construct an \textit{S. mutans} strain which was deficient in lactate dehydrogenase and therefore did not produce lactic acid. In animal trials this strain was found to colonise the tooth surface and displace other mutans streptococci. In preparation for clinical trials this strain was modified to be dependent on dietary \textit{D}-alanine, which allows its removal in the event of unexpected adverse side effects, and to be less capable of transformation, which reduces the possibility of reversion to a lactic acid producing strain.

Guo et al. investigated the use of targeted antisense against \textit{S. mutans}. When \textit{S. mutans} cells were pre-treated with antisense DNA (phosphorothioate-modified antisense oligodeoxyribonucleotides) targeted to the \textit{gtfB} mRNA the cells formed biofilms with reduced biomass, fewer layers, and increased dispersion, without affecting \textit{S. mutans} growth.

Several studies have investigated the anti-caries activity of various peptides. Younson and Kelly designed a synthetic peptide to bind to a cell surface adhesin of \textit{S. mutans} and prevent binding to the tooth surface. The peptide prevented recolonisation of the oral cavity by \textit{S. mutans}. Eckert et al. found an antimicrobial peptide could be targeted to \textit{S.}
*mutans* using an *S. mutans* produced pheromone, the competence stimulating peptide. The combination of the two peptides specifically reduced the levels of *S. mutans* in a biofilm model. Leung et al.\(^\text{253}\) used an antimicrobial peptide active against *A. naeslundii* and found it selectively destabilised the plasma membrane and was able to selectively target *A. naeslundii* in a mixed species biofilm. Guan et al.\(^\text{147}\) found polyaspartate reduced adhesion of *S. sanguis* to hydroxyapatite coated microtitre plates.

Other studies have investigated the use of bacterially produced proteins as antimicrobials against *S. mutans*. Bacteriocins and bacteriocin-like molecules are ribosomally translated proteins that have an inhibitory effect on other, usually closely related bacteria.\(^\text{187}\) They generally target bacteria that are likely to occupy a similar ecological niche. The term bacteriocin-like inhibitory substance (BLIS) has been applied to the bacteriocin-like molecules that are produced by gram-positive bacteria, in particular those that have not been fully characterised.\(^\text{187}\) While *mutans* streptococci have been found to produce many BLIS,\(^\text{27}\) it is less common to find BLIS which target *mutans* streptococci.\(^\text{29}\) However, several studies have screened for BLIS production and found activity against *mutans* streptococci.\(^\text{29,188}\) Mutacin 1140 is produced by some strains of *S. mutans* and is active against a range of *mutans* streptococci, including other *S. mutans* strains, *S. sobrinus* and *Streptococcus rattus*.\(^\text{175}\) Mutacin production can be used to target *mutans* streptococci in the plaque or outcompete non-mutacin producing *S. mutans* strains.\(^\text{83}\) O’Conner et al.\(^\text{329}\) found that lacticin 3147, a lantibiotic produced by *Lactococcus lactis*, reduced *S. mutans* in broth culture and salivary preparations.

A range of “natural” products are also being investigated as potential anti-caries agents. Xanthorrhizol, a turmeric extract, reduced *S. mutans* biofilm formation when it was used to pre-treat the substrata.\(^\text{367}\) Cranberry juice reduced the adherence of *S. mutans* cells when it was used to treat saliva coated hydroxyapatite and inhibited glycosyltransferase activity *in vitro*.\(^\text{233}\) Compounds found in propolis, a bee product, have also been tested. Flavones inhibited glycosyltransferase activity, while flavanones reduced the growth of *S. mutans*.\(^\text{235}\) Chitosan had a high antimicrobial effect against *S. mutans* *in vitro*\(^\text{25,68}\) and significantly reduced plaque formation *in vivo*, however, not to the same extent as 1% chlorhexidine.\(^\text{25}\) It also reduced cellular attachment when used to coated glass pre-treated with human saliva.\(^\text{68}\) Chitosan was also found to act synergistically with chlorhexidine.\(^\text{101}\) Linke and LeGeros\(^\text{269}\) found black tea extract reduced caries formation in a hamster dental caries model.
1.5 Bacterial resistance to antimicrobials

With any antimicrobial treatment, the development of bacterial resistance is a concern. The development of resistance to antibiotics has been investigated most often. Antibiotic resistance has been found in numerous gram positive bacterial strains, and is spread by the horizontal transfer of resistance genes which are often plasmid encoded. Antibiotic resistant bacterial strains lead to difficulties in the treatment of bacterial infections and increased healthcare costs.

Extensive research has been carried out on the development of antimicrobial resistance and how to reduce its occurrence. The use of antimicrobial agents creates an environment with a selective pressure for resistance and the frequency of antibiotic use has been correlated with antibiotic resistance. Exposure to sub-lethal doses of antibiotics also promotes resistance development. The spread of resistance during drug use is more rapid than the decline in resistance when antibiotic use is halted. This is because the fitness cost of maintaining resistance in the absence of antibiotics is generally low, and less than the benefit of resistance in the presence of antibiotics. Antimicrobials should therefore be used at concentrations sufficient to eliminate the target bacteria. However, in the oral cavity antimicrobials are not applied at concentrations that are able to eliminate the cariogenic bacterial population, nor is this desired as the equally affected non-cariogenic oral microflora is generally beneficial. This suggests that use of antimicrobial oral care products will tend to be selective for resistant populations.

Studies have shown daily use of fluoride gels increases the level of fluoride resistant bacteria in the plaque, although this resistance appears to be transient. Streckfuss et al. tested 54 S. mutans isolates from patients receiving daily 1% NaF gels. Thirteen percent of the S. mutans strains exhibited resistance to fluoride, however, all of the strains reverted to fluoride susceptibility after a maximum of seven passages in the laboratory. Resistance to fluoride in S. mutans was induced in vitro by exposure to gradually increasing concentrations of fluoride and this resistance was stable. Strains resistant to fluoride have also been found to have greater sucrose dependent adherence in an in vitro assay. However, other studies have found exposure to fluoride six times a week in a rat caries model did not result in detectable fluoride resistance and that a laboratory induced fluoride strain was unable to outcompete a wild-type strain when both were introduced to the rat model and exposed to fluoride treatments.
Bacterial resistance to xylitol occurs after long term exposure\textsuperscript{305,360,440} and after xylitol exposure halts resistant strains persist in the oral cavity.\textsuperscript{440} Some studies have suggested xylitol-resistant \textit{S. mutans} strains are less cariogenic than xylitol-sensitive strains.\textsuperscript{39,429} However, Assev et al.\textsuperscript{21} found xylitol-resistant strains produced more lactic acid per colony forming unit (cfu) than xylitol-sensitive strains, regardless of the presence of xylitol in the medium.

Resistance to chlorhexidine has been demonstrated \textit{in vitro} when strains are exposed to gradually increasing concentrations.\textsuperscript{105,434} This resistance has been attributed to both inheritable DNA alterations in an \textit{S. sanguis} strain\textsuperscript{473} and transient changes in the cell envelope in a \textit{Pseudomonas} strain.\textsuperscript{430} Changes in the envelope of \textit{Pseudomonas stutzeri} also led to increased resistance to several other antimicrobials.\textsuperscript{430} Deng et al.\textsuperscript{105} found that the ClpP serine protease was upregulated in \textit{S. mutans} during exposure to antimicrobials and was essential in the development of tolerance to chlorhexidine and hydrogen peroxide.

To reduce the development of resistance, the use of antimicrobial combinations has been investigated. The use of combinations of antimicrobials in a single individual reduces the rate at which resistance occurs,\textsuperscript{52} unless resistance genes acquired for both antimicrobials are present on the same plasmid.\textsuperscript{52} This is considered to be more effective than sequential cycling of different antimicrobials.\textsuperscript{47,357}

\textbf{1.6 Antimicrobial combinations}

The use of combinations of antimicrobials can not only help reduce the development of resistance but can give increased inhibition of the target organism. Chlorhexidine acts synergistically with several antimicrobial agents, including fluoride,\textsuperscript{212,280} xylitol\textsuperscript{100} and chitosan.\textsuperscript{101} Meurman et al.\textsuperscript{304} found that total inhibition of \textit{S. mutans} cultures was achieved with 0.5\% fluoride and 0.1\% chlorhexidine when antimicrobials were applied individually but fluoride with chlorhexidine inhibited growth at 0.05\%. Lobos et al.\textsuperscript{273} found chlorhexidine had a synergistic interaction with the bacteriocin PsVP-10 and Filoche et al.\textsuperscript{128} found the combination of essential oils and chlorhexidine was four to ten times more effective than chlorhexidine alone at killing \textit{S. mutans} in a biofilm. Triclosan acts additively with fluoride\textsuperscript{344} and a zinc citrate and triclosan formulation is more effective at inhibiting glycolysis than triclosan alone\textsuperscript{56}.
Koo et al. have investigated the effects of apigenin, a bioflavonoid, and tt-farnesol, an alcohol found in citric essential oils, originally isolated from propolis. Individually apigenin inhibited the activity of GTFs, while tt-farnesol reduced the growth of *S. mutans* and *S. sobrinus* biofilms, and in combination they reduced smooth-surface caries in a rat model. It was found the combination of apigenin, tt-farnesol and fluoride was more effective at reducing caries in a rat model than any of the agents alone or in pairs and the reduction in caries was similar to that seen with the chlorhexidine and fluoride, used as a positive control.

Improved inhibition of *S. mutans* or dental caries has also been achieved by using a combination of treatment strategies. Several studies have shown the use of an antimicrobial mouthwash after brushing with toothpaste is more effective at reducing plaque compared to toothpaste alone. Särner et al. found approximal fluoride concentrations could be enhanced with the use of multiple fluoridated products and that the order in which the products were used impacted on the concentrations of fluoride achieved. Modesto et al. found cells treated with xylitol after multiple chlorhexidine exposures were less able to form biofilms *in vitro* compared to cells only treated with chlorhexidine or xylitol.

### 1.7 Zoocin A

Zoocin A is a D-alanyl-L-alanine endopeptidase produced by *Streptococcus equi* subspecies *zooepidemicus* 4881. *S. equi* subspecies *zooepidemicus* 4881 is a Lancefield group C streptococci, commonly found in a range of animal hosts, but rarely in humans. *S. equi* subspecies *zooepidemicus* 4881 was originally isolated from an equine origin and its activity against *S. mutans* was first reported by Schofield and Tagg. S. *equi* subspecies *zooepidemicus* 4881 was found to have activity against a range of mutans streptococci in a deferred antagonism test, including all *S. mutans*, *S. sobrinus* and *Streptococcus cricetus* strains tested. However, none of the *S. rattus* or *S. equi* subspecies *zooepidemicus* strains tested were sensitive. Most *S. anginosus* and *S. gordonii* strains tested were also sensitive, whereas the majority of the *S. salivarius*, *S. oralis* and *S. sanguis* strains were resistant. Simmonds et al. purified zoocin A from the supernatant of *S. equi* subspecies *zooepidemicus* 4881 cultures, obtaining titres of up to 64 arbitrary units (AU) per millilitre, and identified zoocin A as a protein of 30 kDa. Similar inhibition patterns were seen with purified zoocin A compared to the deferred antagonism test, although the relative
sensitivity of the different strains was found to vary. This is probably because the threshold for detection of sensitivity by deferred antagonism is an MIC of around 6 μg/ml. Zoocin A was found to be heat labile, it was relatively stable at 4 and 37°C, however activity was rapidly lost at 60°C. It was also found to be stable over a range of pH concentrations, especially under alkaline conditions. Zoocin A activity was assayed after exposure to solutions with the pH ranging from 3.3 to 10, for up to 24 hours. Activity was only lost rapidly at a pH of 3.3. The effect of a range of enzymes on zoocin A was tested. Lipase, deoxyribonuclease 1, papain, catalase and α-amylase had no effect on protein activity, while trypsin, protease and α-chymotrypsin resulted in rapid decrease in activity. Zoocin A was shown to act by rupturing the cell wall by cleaving the peptidoglycan cross-links, causing separation of the cell membrane from the cell wall and loss of the cytoplasmic constituents.

Cloning of and sequence analysis of the zoocin A gene (zooA) revealed zoocin A is translated as a 285 amino acid precursor protein, from which a 23 amino acid leader sequence is cleaved to yield the functional protein. Zoocin A was also found to contain a conserved N-terminal domain, a linker sequence and C-terminal domain, a common arrangement for peptidoglycan hydrolases from gram positive bacteria. The N-terminal domain had high homology to the N-terminal region of lysostaphin. Lysostaphin is a lytic molecule active against S. aureus, which cleaves the pentaglycine crosslink within the peptidoglycan molecule. The C-terminal domain of lysostaphin is thought to be involved in cell wall binding due to its homology with the C-terminal domain of N-acetylmuramyl-L-alanine amidase, an S. aureus peptidoglycan hydrolase. The C-terminal domain of zoocin A does not share homology with lysostaphin, which may be expected as the proteins differ in their target species. Recombinant domain analysis of zoocin A demonstrated that the N-terminus of zoocin A contains the functional groups for hydrolysis of the peptidoglycan cross-links and that the C-terminal region was required for cell binding of zoocin A and is probably required for optimal protein activity. This two domain model was confirmed by nuclear magnetic resonance (NMR) analysis. It was later found by Gargis et al. that zoocin A is a D-alanyl-L-alanine endopeptidase and its activity therefore resulted in peptidoglycan lysis. Zoocin A was also found to be a penicillin-binding protein, capable of both binding and cleaving penicillin and penicillin inhibited the peptidoglycan hydrolase activity of the enzyme. Zoocin A is also a metalloenzyme, binding a single Zn^{2+} ion at the active site. There are differences in susceptibility to zoocin A between closely related strains. Strains sensitive to zoocin A were all found to have peptidoglycan with di- or tri-alanine cross-links
and it was suggested that resistance to zoocin A may be due to differences in cell wall cross-linking, resulting in an inability of zoocin A to cleave the cell wall, especially as the resistant *S. oralis* and *Lactococcus* strains contain direct covalent or single d-Asp amino acid cross-links.\(^\text{391}\) However, this cannot be the case for all resistant strains. Simmonds et al.\(^\text{391}\) demonstrated *S. rattus*, which is resistant to zoocin A, had similar cell wall cross-linking to *S. mutans*, which indicated there must be other mechanisms of resistance. The sensitivity of bacterial strains to zoocin A has been shown to correlate with the amount of zoocin A bound by the cells, indicating the ability to bind to the target is a major factor in susceptibility.\(^\text{8}\) The difference in susceptibility between closely related strains may be due to the differences on the cell surface. *S. mutans* and *S. rattus* are closely related, however *S. mutans* binds zoocin A more effectively than *S. rattus*. This may be due to the presence of teichoic acid on the cell surface of *S. rattus*. Removal of the cell wall polymers from *S. rattus* increased the binding of zoocin A to the cell surface.\(^\text{8}\) The resistance of *S. oralis* was also linked to reduced binding of zoocin A. Akesson et al.\(^\text{8}\) suggested this may be due to the presence of choline in the cell wall. Zoocin A was able to bind, yet not cleave purified *S. oralis* peptidoglycan, suggesting binding was not the only factor involved in resistance. It has also been suggested resistance to zoocin A may be related to the production of protease production in lactococcal strains.\(^\text{8}\)

Producer cell immunity to lysostaphin is achieved by expression of the *lif* gene (also called *epr*), which changes the peptidoglycan cross-links, resulting in more serine and less glycine residues.\(^\text{102}\) *Lif* is very similar to *femAB*, genes essential for methicillin resistance in *S. aureus*.\(^\text{44,436}\) Beatson et al.\(^\text{37}\) found zoocin A resistance of the producer strain is mediated by *zif* (zoocin A immunity factor), a gene with high homology to *fem* and *lif*, and insertion of *zif* into sensitive strains of *S. gordonii* conferred resistance. However, initial analysis of peptidoglycan purified from *S. equi* subspecies *zooepidemicus* and *S. gordonii* strains showed no difference between zoocin A immune and sensitive strains, or strains with and without *zif*, so the gene product Zif (unlike the gene products of *femAB* and *lif*) did not appear to result in a direct change in the synthesis of the peptidoglycan.\(^\text{37}\) Strains containing a functional *zif* gene had cell walls that could not be hydrolysed by zoocin A, even at high concentrations,\(^\text{247}\) however, in lysostaphin resistant cells *lif* expression left a proportion of the cells sensitive.\(^\text{163}\) A comparison of wild type *S. equi* subspecies *zooepidemicus* 4881 with a *zif* knockout strain showed *zif* expression resulted in significantly less zoocin A bound to the cell surface, although sufficient zoocin A bound to the *zif* expressing cells to suggest the immunity of the producer organism was due to prevention of peptidoglycan hydrolysis.\(^\text{8}\) Farris et al.\(^\text{127}\)
inserted the lysostaphin resistance gene into *S. equi* subspecies *zooepidemicus*, strain 8b (a strain that is sensitive to, and does not produce, zoocin A) and found this increased the zoocin A resistance of the strain. Analysis of the peptidoglycan found an increase in the levels of serine in the cross-linkages, thought to be responsible for the observed increase in zoocin A resistance. Further analysis of the *zif* gene found that Zif had a high degree of similarity to MurM and MurN, proteins involved in the addition of amino acids to the peptidoglycan cross-linking. Analysis by mass spectrometry of peptidoglycan from *zif* containing strains determined a higher proportion of peptidoglycan cross-bridges contained an additional l-alanine, which resulted in reduced zoocin A binding and resistance to zoocin A.

Lai et al. cloned *zooA* into pQE-80L in *E. coli* M15 containing pREP4 and were able to produce ten times more zoocin A than was obtained by the concentration of the supernatant of *S. equi* subspecies *zooepidemicus*. The recombinant zoocin A had the same activity as native zoocin A in the deferred antagonism test, indicating the polyhistidine-tagged (histagged) recombinant protein did not have altered biological activity. Addition of zoocin A to mid-log-phase cultures of *S. pyogenes* resulted in a rapid decrease in both the optical density (OD) and the cell viability. Conversely, in *S. mutans* 10449 cell viability was not reduced until over three hours after the exposure to zoocin A and the OD did not reduce, although it stopped rising as soon as the zoocin A was added. However, these effects were probably dose related, rather than due to a difference in mode of action. Simmonds et al. set up a triple species biofilm model with *S. sanguis* 209, *A. viscosus* A69 and *S. mutans* NCTC. BHI broth was dropped onto hydroxyapatite discs, at 12 ml/h for four hours, before the addition of approximately 8 μg zoocin A. After 2 or 30 minutes the BHI flow was restarted for a further 20 hours. It was found that *S. mutans* NCTC was reduced by approximately two logs and this reduction was maintained for up to 20 hours post zoocin A exposure. The levels of *S. sanguis* 209 and *A. viscosus* A69 were not affected immediately after zoocin A exposure, however after 24 hours the levels of *S. sanguis* 209 were significantly lower than the control. They also found zoocin A incubated in sterilised whole saliva had no reduction in activity, indeed after 24 hours at 37°C the zoocin A in saliva appeared to be stabilised compared to the buffer control. No increase in activity occurred in samples from 20 minutes to 3 hours when compared to the control, so this result was likely to be due to stabilisation of zoocin A against thermal degradation by a salivary component.
1.8 Monolaurin

Monolaurin (glyceryl monolaurate) is a glycerol monoester of lauric acid. The antimicrobial properties of fatty acids and their corresponding esters have been investigated over the past fifty years. Esters have been found to be more effective than fatty acids\textsuperscript{34,202} and monolaurin has been shown to be the most effective monoglyceride tested.\textsuperscript{91,203} Monolaurin is a saturated fatty acid with 15 carbon atoms. Both the degree of saturation\textsuperscript{202,323} and length of the carbon chain\textsuperscript{91,203} influences the antimicrobial effects of the fatty acids. The optimum chain length is between 12 and 16 carbon atoms.\textsuperscript{203} Kelsey et al.\textsuperscript{215} found that the saturated fatty acids and their esters inhibited the total numbers of \textit{S. aureus}, while poly-unsaturated compounds increased in the lag phase of bacterial growth.

Monolaurin is active against a wide range of bacterial species. It is particularly active against gram-positive bacteria, including Group A and β-haemolytic streptococci,\textsuperscript{202,376} \textit{Streptococcus pyogenes},\textsuperscript{34} \textit{S. aureus},\textsuperscript{202,215} \textit{S. epidermidis},\textsuperscript{34,91} \textit{Nocardia asteroides},\textsuperscript{91} \textit{Bacillus subtilis},\textsuperscript{498} \textit{Listeria monocytogenes},\textsuperscript{464} a variety of \textit{Micrococcus}, \textit{Enterococcus} and \textit{Corynebacterium} species.\textsuperscript{74,91} It is also active against several oral bacteria\textsuperscript{159} including \textit{S. mutans},\textsuperscript{203} \textit{S. sanguis}\textsuperscript{159} and \textit{A. viscosus}.\textsuperscript{201} Monolaurin is also active against \textit{Mycobacterium terrae}\textsuperscript{351} and yeast species such as \textit{Candida albicans}.\textsuperscript{45,202} Although many studies indicate monolaurin is more active against gram-positive bacteria, it is also active against several gram-negative bacteria such as \textit{Helicobacter pylori}\textsuperscript{341,422} and some \textit{Enterobacter} species.\textsuperscript{74} Some studies have shown activity against \textit{E. coli},\textsuperscript{456,498} while other studies have found no effect.\textsuperscript{351,376} When combined with a cation chelator monolaurin is more active against gram-negative bacteria.\textsuperscript{34}

Monoglycerides isolated from the fungus \textit{Sepedonium ampullosporum} were noted to have antitumour effects\textsuperscript{209} and further studies noted monolaurin was particularly active against a tumour model, keeping the mice tumour-free.\textsuperscript{210} Monolaurin suppressed ruminal methanogenesis in a rumen model with certain basal diets\textsuperscript{226} and suppressed growth of bacteria isolated from skin infections.\textsuperscript{74} Monolaurin has also been found to be an antiviral agent. Ten- and twelve-carbon fatty acids were active against visna virus,\textsuperscript{176} respiratory syncytial virus and human parainfluenza virus.\textsuperscript{177} Li et al.\textsuperscript{257} found that monolaurin prevented simian immunodeficiency virus transmission in a macaque model and \textit{in vitro} it was shown to inhibit mucosal signalling. Monolaurin has been investigated as a food
preservative and was effective against *E. coli*, *S. aureus* and *L. monocytogenes* in milk.\textsuperscript{300,464} However, the activity of monolaurin was dependent on the amount of fat in the test medium. Wang et al.\textsuperscript{464} found monolaurin could inhibit *L. monocytogenes* growth in skim milk and not in whole milk, and that the amount of inhibition decreased with increasing fat content in milk. They suggested this was due to fat globules or lipophilic proteins sequestering the monolaurin.\textsuperscript{464} Glass and Johnson\textsuperscript{142} found the presence of 20% fat significantly reduced the antimicrobial effects of free fatty acids against *Clostridium botulinum*. Projan et al.\textsuperscript{352} found monolaurin interferes with the signal transduction required to initiate toxin production in *S. aureus* and proposed monolaurin inserts itself into bacterial membranes to interfere with signalling proteins, or causes a conformational change in transmembrane proteins. Schlievert et al.\textsuperscript{376} found exotoxin production in streptococci was inhibited by monolaurin.

The exact mode of action of monolaurin remains unclear and is probably due to a combination of factors. It is generally agreed monolaurin acts upon the cell membrane, possibly affecting transport into the cell\textsuperscript{134} or signal transduction.\textsuperscript{370} It has been suggested that this is due to the surfactant nature of monolaurin.\textsuperscript{203} However, other surfactants, such as sodium dodecyl sulphate (SDS), have reduced antimicrobial activity compared to monolaurin, so other mechanisms of action must be involved.\textsuperscript{202} Fatty acids increase the permeability of the plasma membrane\textsuperscript{146} and studies using electron microscopy found that monoesters disrupt the plasma membrane of streptococci\textsuperscript{46} and *C. albicans*.\textsuperscript{45} Examination of *L. monocytogenes* cells treated with 50 μg/ml monolaurin showed the cytoplasm separating from the cell wall in some cells, and lysis of the cell and loss of cytoplasmic contents in others.\textsuperscript{464} Dodecylglycerol (a twelve carbon monoglyceride) inhibited peptidoglycan synthesis in two streptococcal species\textsuperscript{455} and stimulated a protease, which activates an autolysin that degrades peptidoglycan.\textsuperscript{454} Streptococci are more susceptible to monolaurin than staphylococci, possibly due to staphylococcal production of a lipase that degrades the monolaurin,\textsuperscript{370,376} or differences in the peptidoglycan cross-linking of the different species.\textsuperscript{46}

The antimicrobial activity of monolaurin against a wide range of bacteria gives it potential as a therapeutic agent. It is more active in slightly acidic environments, making skin (pH 5.5) and saliva (pH 6.0-6.5) environments where monolaurin can be effective.\textsuperscript{203} Monolaurin also has synergistic effects with a range of other compounds. Zhang et al.\textsuperscript{498} found monolaurin acted synergistically with nisin and sodium dehydroacetate against *E. coli*, *S. aureus* and *B. subtilis*, and with ethylenediaminetetraacetic acid (EDTA) against *E. coli* and *B. subtilis*. McLay et al.\textsuperscript{300} found a combination of the lactoperoxidase system and monolaurin had a
synergistic effect on *E. coli* O157:H7 and *S. aureus* in milk. Other compounds that act synergistically with monolaurin include monocaprin and lauric acid.34

Lauricidin is a distilled monoglyceride extracted from coconut oil stock. It consists of 90% monolaurin. Lauricidin has generally regarded as safe (GRAS) status and is used as a food preservative. It is also available commercially as an antimicrobial dietary supplement. Lauricidin has been investigated as an anti-caries agent201 and several studies have used lauricidin to investigate the effects of monolaurin against a range of bacteria.60,117,142

1.9 Delivery systems for antimicrobials

Any antimicrobial treatment must be effectively delivered to the oral cavity. A number of delivery systems have been used to medicate the oral cavity. These include toothpastes, mouthwashes, gels and chewing gums.

Toothpastes are an excellent delivery system for antimicrobial agents and frequently contain fluoride.265,415,418 Diamanti et al.107 found higher concentrations of fluoride in toothpastes were more effective at reducing demineralisation and promoting remineralisation in an *in vitro* model and fluoride containing toothpastes reduced caries incidence in clinical trials.36,327,418 However, several other anti-plaque agents, such as chlorhexidine,198 are incompatible with toothpaste components.416 Anionic surfactants found in toothpaste formulations interact with chlorhexidine, which reduces its effectiveness.198 Triclosan maintains its antimicrobial activities when included in toothpaste416 and enhances the activity of toothpastes containing zinc citrate416 and fluoride.344,457 Brading et al.56 found that a toothpaste formulation containing a zinc salt and triclosan was able to inhibit glycolysis and prevent the pH drop following sugar challenge in an *in vitro* model.

Mouthwashes are another common delivery system and can contain a range of active ingredients, including fluoride,113,115,468 essential oils,79,346,478 xylitol100,169 and other herbal151,277 and chemical319,389 compounds. Chlorhexidine is a common component of mouthwashes.113,438,453 Löe and Schiøtt found that two daily mouthwashes of 0.2% chlorhexidine gluconate prevented plaque formation and gingivitis, but noted that a single rinse was not sufficient, suggesting that rinsing did not always reach all tooth surfaces effectively.274 Therefore chlorhexidine must be applied in a way that reaches all tooth surfaces.274 Chlorhexidine is thought to bind to bacteria, salivary proteins and the tooth
surface and be maintained in the oral cavity for at least 24 hours following use in a mouthwash. Jenkins et al. found that a 0.2% chlorhexidine mouthwash reduced the salivary bacterial count for at least seven hours following use, whereas a variety of toothpaste formulations reduced the bacterial count for a maximum of five hours.

Antimicrobial agents can also be applied to the tooth surface in the form of gels or varnishes. These are sustained release delivery devices, which have a longer tooth contact time and thus may facilitate higher concentrations of the active compound entering the plaque and better biofilm penetration. Varnishes and gels often contain fluoride or chlorhexidine, although other compounds such as cetylpyridinium chloride have been used. Although studies on varnishes and gels have found they reduce salivary mutans streptococci and increase fluoride uptake, they generally do not show a caries preventative effect or increased remineralisation.

Medicated chewing gums are another delivery system for the oral cavity. Chewing gum is kept in the mouth for an extended period of time and can result in prolonged drug release and contact with the teeth. Chewing gum offers the added advantage of increasing salivary secretion when chewed. This increases the plaque pH, and improves the protective properties of saliva such as mineral supersaturation and antimicrobial activity, thus reducing the effects of cariogenic bacteria. Chewing sugar-free gum after meals can also promote the remineralisation of enamel and chewing gums are a commonly used commodity which are readily accepted by patients, improving compliance. Oztas et al. found use of a fluoride containing gum increased salivary fluoride levels and studies on chlorhexidine containing gum found its use resulted in significantly lower levels of plaque. However, chlorhexidine gum has a bitter taste and results in reversible tooth staining and taste impairment. Na et al. incorporated an antimicrobial decapeptide into a traditional chewing gum and found chewing resulted in a sustained release of the peptide into saliva over 20 minutes which is considered the average time for gum chewing.

1.10 Plaque models

Due to the cost and ethical issues surrounding human studies, models of dental plaque are required to investigate the etiology of dental caries or potential caries treatments. These can range from simple pure culture studies to complex artificial mouth systems with electronic monitoring and control.
1.10.1 Culture models

The simplest models involve a pure culture of an oral bacterial species. These are most often used in preliminary studies to investigate the growth inhibitory effects of potential antimicrobials, and can provide information on the antimicrobial’s mode of action and the susceptibility of an individual species. However, growth inhibition does not necessarily equate to effectiveness in vivo, as these systems do not take into account environmental conditions such as nutrient levels, pH, or the presence of other bacterial species. To model the effects of bacterial interaction a co-culture can be used, where several species of bacteria found in the oral cavity can be cultured together, and the effects of an antimicrobial on the mixed populations can be investigated. The interactions between bacterial species in a co-culture environment can have a significant impact on antimicrobial effectiveness. Co-culture models can be very complex. Bradshaw et al. used a continuous culture model containing up to ten different species to model shifts in the oral microflora following sucrose exposure in the presence and absence of fluoride and to investigate the effects of triclosan and zinc citrate on the oral microflora.

1.10.2 Biofilm models

Other bacterial activities such as biofilm formation can also affect the action of an antimicrobial. Bacterial susceptibility to antimicrobials is often reduced in biofilms and in established biofilms their inhibitory effect is limited to superficial layers. A biofilm model aims to simulate the oral environment in the laboratory and allows investigation into the effects of microbial interaction and diffusion limitation on antimicrobial efficacy. There are many different biofilm models, but the basis of them all is to supply nutrients in a continuous or intermittent manner to plaque bacteria growing as a biofilm on a substrate under conditions that mimic those of the oral environment. Simpler biofilm models usually involve growing a biofilm on a glass or plastic substrate surrounded by a nutrient broth, while complex models use hydroxyapatite or sterilised human teeth as a biofilm surface and a complex artificial saliva. They often contain multiple chambers to allow replicates or comparisons and real time monitoring of conditions, such as culture pH, is possible.
There is a huge range in biofilm models, with variations in the number and type of bacterial species used, nutrient source, frequency of sugar exposure, biofilm substrata and incubation conditions and length. Biofilm models need to find a balance between realistic modelling of the *in vivo* environment and practicality, simplicity and ease of use, and it is important that the model used is able to provide meaningful data. The biofilm model must therefore be appropriate for the type of experiments being carried out. Biofilm models need to be easy to sterilise and allow aseptic handling of samples, which should be removable (complete with the substratum) to prevent disturbance of the biofilm and allow for microscopic examination if required. The samples need to be discrete and reproducible.

The simplest biofilm models are single species biofilms grown on glass slides or microtitre plates. Altman et al. used a single species biofilm model grown on glass to demonstrate the effectiveness of an antimicrobial peptide against *S. mutans*, although bacteria in a biofilm were less susceptible to the peptide than planktonic bacteria. A single species biofilm addresses whether the antimicrobial will penetrate a biofilm, but does not take microbial interaction into account. Multispecies biofilms have been shown to be more resistant to antimicrobial agents than single species biofilms. Kara et al. used single and dual species biofilms in a microtitre plate to investigate the actions of chlorhexidine against an oral biofilm. In a dual species biofilm of *S. mutans* and *Veillonella parvula*, bacteria were more resistant to the effects of chlorhexidine compared to single species biofilms and *S. mutans* was found to be more susceptible than *V. parvula* in the mixed biofilm.

The Zurich model described by Guggenheim et al. is a multispecies batch culture system where the biofilms are grown in 24-well culture plates. Hydroxyapatite discs are placed in the wells to act as a substrate for the biofilm. The discs are coated in sterilised saliva, covered in growth medium and inoculated with a mixed bacterial inoculum. The growth medium is changed every 24 hours. The Zurich model has been shown to be stable and reproducible after 40 and 65 hours incubation and has been used to examine remineralisation and demineralisation of enamel, as well as the effect of several antimicrobial agents on biofilms. An advantage of the Zurich model is that antimicrobial agents can be added to the biofilm and then removed after precise time intervals, whereas in flow cell cultures the flow rate and culture volume determine the rate of removal of antimicrobials added to the system. The biofilms can also easily be removed from the culture plates for further examination, including cell counts and microscopy. The samples...
are also truly independent and experiments could be carried out on any substrate. However, the Zurich model is a sedimentation model where the biofilms are constantly submerged in nutrient media, unlike in the oral cavity.

Flow cell models grow biofilms under a continuous flow of media. There are many variations on a flow cell, however, they generally consist of a base with parallel chambers covered by a glass cover slip. The parallel chambers allow independent replicate biofilms to be grown in the same flow cell. The flow cell is connected to a nutrient reservoir, pump, bubble trap and effluent container. A flow cell is designed to be compatible with examination under a microscope, which allows in situ examination of the biofilm throughout the experiment. Confocal laser scanning microscopy allows optical sectioning of the flow cell biofilm and the assembly of 3-D images. Many studies have used flow cells to investigate biofilm formation. Filoche et al. used a flow cell to investigate the formation of multispecies biofilms and found the formation of multispecies bacterial aggregates was important in biofilm development, proliferation and the reduced susceptibility of the biofilm to antimicrobial agents. Deng et al. grew cells that were constitutively expressing green fluorescent protein in a flow cell and used the fluorescence as a metabolic indicator to investigate changes in fluorescence after glucose challenge or exposure to antimicrobials found in oral care products. They found the increase seen in fluorescence after exposure to glucose could be prevented by the addition of antimicrobial agents such as chlorhexidine, sodium fluoride, hydrogen peroxide and sodium chloride and that this inhibition was dose dependent.

A more complex biofilm model is the constant depth film fermentor (CDFF), which is a frequently used biofilm model. It contains up to 75 recesses, called plugs, in which the biofilms are grown on the surface of choice. The depth of the plugs can be set to determine the maximum thickness of the biofilms, above the top of the plug excess biofilm is scraped off by a rotating scraper bar. This bar also distributes the nutrient media, which is dripped onto the turntable directly in front of the scraper and then spread over the biofilms. Waste media flows off the turntable and is collected from an outlet port in the base plate. The CDFF allows multiple replicates in one run but does not allow different conditions to be trialled in the same run. The biofilm replicates may not be independent from each other as the same scraper operates over all the biofilms, which would be expected to lead to cross-contamination. However, the CDFF does allow for long term experiments so the biofilm can reach a steady state. The CDFF has been used in a number of biofilm studies.
including to investigate the effect of antimicrobials on a biofilm model. Wilson et al. used a CDFF to investigate the effects of 0.2% chlorhexidine on mixed species biofilms grown with and without sucrose. Exposure to chlorhexidine for one minute decreased the viable count of the biofilms by less than one log, mainly by reducing the streptococci and actinomyces. Pratten et al. also found exposure to 0.2% chlorhexidine for five minutes had a limited effect on multispecies biofilms grown in a CDFF.

A feature of the dental biofilm is its high density and diversity of microbial species. To model the diverse bacterial species in the oral cavity, studies have used a defined bacterial inoculum containing a large number of bacterial species, such as the continuous culture studies by Bradshaw et al. These multispecies cultures were also used in a biofilm model. The continuous culture of nine defined species was allowed to reach a steady state and was used to inoculate a multispecies biofilm model grown in a CDFF. This model was used to investigate the effects of chlorhexidine and examine pH gradients in the biofilm. Microcosms are also used to model the ecological complexity of the oral cavity. Microcosms are laboratory systems that are derived from natural systems in an attempt to simulate those in the natural environment. They can vary in complexity. Filoche et al. developed a simple plaque microcosm by filter sterilising mixed stimulated saliva from donors and using this to inoculate a microtitre plate. From a single pooled saliva donation reproducible biofilms could be made. However, there was always individual variation between different saliva samples collected. Sissons et al. developed a multi-station dental plaque microcosm, an artificial mouth that allows five simultaneous and independent microcosms to be incubated. The artificial mouth consists of multiple stations in the same chamber, ensuring all biofilms are exposed to the same environmental conditions. The biofilms are grown on circular plastic cover slips. Each station has separate lines for biofilm sampling, monitoring of pH and addition of antimicrobials to the biofilms. The delivery of nutrients, gas and other reagents can be computer controlled and real-time pH data can be collected. It is possible to sequentially sample each replicate without introducing contamination. Biofilms grown in the artificial mouth were viable for up to six weeks. The artificial mouth has been used to study plaque growth rates and the pH changes following sucrose challenge. It was found the pH profiles of the artificial mouth biofilms were similar to those seen in vivo and that the magnitude and duration of the decrease in pH was dependent on the mass and thickness of the biofilm and the media flow rate.
The ability to simulate the oral cavity in vitro allows investigations into the complex interactions between oral bacteria and gives a more realistic insight into the effectiveness of possible anti-plaque agents than pure or co-cultures.396,428

1.10.3 Animal models

Animal models can allow analysis of pathogenesis of human periodontal diseases and various treatments. However, care must be taken to ensure a similarity between the animal model and human anatomy and disease.469 Non-human primates are phylogenetically similar to humans and their oral structure and composition is also similar.75,469 For this reason they are often used for the study of periodontal diseases.75,469 Rats are most frequently used for the study of calculus and caries,469 and hamsters have similar dentition to rats and are used for similar purposes.221,269 The ability to produce germfree rats and hamsters make them particularly valuable as they can be infected with human oral bacteria to study dental caries development.403,497 A gnotobiotic rat model has been used to compare the cariogenicity of wild type S. mutans with a mutant strain without the spaP gene, the product of which is involved in attachment. It was found rats infected with the mutant strain had decreased caries status.93 Rat models are used to determine the cariogenic and erosive potential of foods and drinks.95 They have also been found to be valid for the testing of anti-caries treatments, as the responses to fluoride treatment in rats were similar to those observed in humans.419 Thurnheer et al.437 used a rat caries model and an in vitro biofilm model to establish that soluble starch was less cariogenic than sucrose or glucose.437 Rats are also used for immunological studies into dental caries vaccines, where rat caries models are used to test the ability of a vaccine to reduce the incidence of dental caries.109,260,324 Rabbit and primate models are also used to test vaccines, especially when examining the immunological responses to the vaccine.196 Animal models allow investigation into the effects of diet on dental caries216 and research utilising smaller laboratory animals can have considerable advantages over clinical trials as they are simpler and more control can be exerted over variables. However, limitations exist as no animal can be expected to respond to stimuli in exactly the same way as humans.469
1.10.4 Human studies and trials

Clinical studies with human volunteers can be difficult due to problems with volunteer compliance and the length of time often involved. They are also restricted to agents which have been approved for use in humans. However, clinical trials overcome the problems posed by lack of similarity between the in vivo situation and that in animal models or laboratory model systems.

Intra-oral models involve the temporary attachment of a piece of dental hard tissue in the subject’s oral cavity. There it is exposed to the individual’s saliva and microflora as well as any effects of diet and oral care. It can then be removed at the end of the study for in-depth examination that could not be carried out in situ. Many studies have used intra-oral models, especially to examine in situ remineralisation and demineralisation of enamel.

Sjögren et al. used an intra-oral model to evaluate the effects of chewing gum containing fluoride or urea. Demineralised enamel and dentin blocks were bonded to the lower canines and first premolars for each of six four-week trials, in which subjects used chewing gum with fluoride, urea, a placebo, or used no gum. After each trial the discs were removed for analysis by transversal microradiography. It was found that chewing sugar-free gum was enough to prevent further demineralisation of the enamel or dentine. No difference in mineralisation was seen between the three chewing gums.

Studies in human volunteers are also used to study the effectiveness of various oral care protocols and antimicrobial treatments, including mouthwashes, varnishes, fluoride treatments, different toothpaste formations and medicated chewing gums.

1.11 Protein production and purification

To use a protein as an antimicrobial it must be able to be produced in large quantities. Recombinant E. coli is often used for protein production due to its well characterised system. Expression of recombinant protein in E. coli has been used to produce sufficient concentrations of protein for characterisation. Lai et al. used recombinant zoocin A produced in E. coli to characterise the protein and Mosbah et al. used recombinant protein produced in E. coli to characterise the extracellular lipase of Staphylococcus xylosus. Several expression systems have been developed for E. coli, which utilise plasmid expression vectors. There are many different plasmid vectors available, but they contain the same
basic features. The vectors contain the gene to be expressed under the control of a strongly regulated promoter, associated transcriptional and translational controls, the plasmid origin of replication and an antibiotic resistance gene.\textsuperscript{290,406} The plasmid origin of replication also controls the copy number of the plasmid.\textsuperscript{290} There have been conflicting reports on the optimum plasmid copy number. While some studies have found a high copy number beneficial, in others it has negatively impacted on protein yield.\textsuperscript{262,309} The antibiotic resistance gene allows easy maintenance and selection of plasmid containing cells.\textsuperscript{290,406} The promoter used to control the expression of the recombinant protein expression should be strong to maximise protein expression.\textsuperscript{290} There are many promoters, including \textit{lac}, \textit{tac} and \textit{trc} which are induced by the addition of isopropyl-β-D-thiogalactoside (IPTG).\textsuperscript{145,252,297,485} Other promoters can be constitutive or controlled by a thermosensitive repressor.\textsuperscript{35,135,195} Inducible promoters should also have low levels of basal transcription as maximum protein expression is usually achieved by growing the cells to a high cell density before inducing protein expression.\textsuperscript{290,406} This also reduces any toxic effect the recombinant protein may have on the producing strain\textsuperscript{65,111,486} and incomplete repression can cause plasmid instability, decreased growth rate and decreased protein yield.\textsuperscript{43,303} A commonly used inducible promoter system is based on the \textit{lac} operon.\textsuperscript{145} A strong promoter is repressed by the binding of a repressor protein, encoded by \textit{lacI}, to the \textit{lac} operator, which prevents transcription occurring.\textsuperscript{406} A mutant of \textit{lacI}, \textit{lacIq}, is frequently used as it results in ten times greater expression of the repressor protein and gives tighter expression regulation.\textsuperscript{70,406} This repression is removed by the introduction of IPTG which binds and inactivates the \textit{lac} repressor protein.\textsuperscript{406}

It is commonly reported that the presence of plasmids can have a negative effect on bacterial growth rate and yield. It is thought this is due to an increase in the use of cell resources to maintain the plasmid and plasmid-related activities.\textsuperscript{16} Andersson et al.\textsuperscript{16} investigated the effects of plasmid presence, chemical induction and cellular stress responses on bacterial growth and yield. Induction of a plasmid encoded protein with IPTG was found to significantly reduce the final biomass of the culture, while high plasmid content only reduced the final biomass slightly.\textsuperscript{16} The expression system used resulted in a relatively low amount of recombinant protein, suggesting most of the effects upon cell growth were due to the IPTG used to induce the cells.\textsuperscript{16} Previous studies have shown that the addition of IPTG to \textit{E. coli} results in the expression of several stress response proteins\textsuperscript{238} which are thought to have a detrimental effect on final biomass and protein yield.\textsuperscript{16} Hasenwinkle et al.\textsuperscript{157} found that in a shake flask culture, addition of 1 mM IPTG had no effect on the growth rate or final cell
density of the culture, however, they suggest that the effect of IPTG upon cells at high density may not reflect those found in low density shake flasks.\textsuperscript{157} Most recombinant proteins are accumulated intracellularly in \textit{E. coli} making productivity proportional to the final cell density.\textsuperscript{251} Research has therefore aimed to improve productivity by increasing the cell density to which cells are grown. To this end, high cell-density cultures have been developed for \textit{E. coli}, which result in high volumetric productivity, reduced culture volume, reduced equipment, improved downstream processing and thus lower costs.\textsuperscript{251,387}

1.11.1 Codon usage

\textit{E. coli} codon bias is one factor which can reduce protein yield. For each amino acid \textit{E. coli} uses certain codons more frequently. These major codons occur more often in highly expressed genes, whereas rare codons are more likely to be utilised in genes expressed at a low level.\textsuperscript{205} The frequency at which a codon is used has been shown to correlate with the levels of the appropriate tRNA.\textsuperscript{183,189,371} Therefore the codon composition of recombinant genes can affect protein production.\textsuperscript{205,406} A high level of rarely used codons in the recombinant gene can result in reduced protein yield, as the ribosome stalls at the rarely used codon due to a limited supply of the appropriate tRNA. This can also lead to translational errors in the recombinant protein.\textsuperscript{267,301} In \textit{E. coli} the rare arginine codons AGG and AGA have been identified as particularly likely to impact on recombinant protein expression.\textsuperscript{61,80,205} Other codons which have impacted on protein production in some experimental systems are CUA, AUA, CCC, CGG and CGA.\textsuperscript{94,143,205,296} To overcome these problems codon optimisation is carried out.\textsuperscript{145} This can be achieved by site-directed mutagenesis to alter rare codons to those used more frequently in \textit{E. coli}.\textsuperscript{179,206} Calderone et al.\textsuperscript{69} used site-directed mutagenesis to alter three rare AGA codons in a eukaryotic fusion protein expressed in \textit{E. coli} and found this prevented the translational errors that were seen when the rare codons were present. An alternative approach is to co-transform \textit{E. coli} with a plasmid which encodes the rare codon tRNA.\textsuperscript{24,219} Dieci et al.\textsuperscript{108} found using a plasmid containing \textit{argU}, which encodes the tRNA for the AGA and AGG codons, improved the yield of five different eukaryotic proteins.

1.11.2 Large-scale protein production

While most recombinant protein production for laboratory experiments is carried out in small volumes (fewer than five litres), for some applications large amounts of protein are needed.
To produce sufficient protein larger culture volumes are required. A fermentor can incubate larger volumes, however, it has reduced mixing efficiency compared to cultures in shake flasks, a problem which increases with increased fermentor size.\(^{167}\) This leads to chemical gradients in large scale fermentors\(^{488}\) and cells close to nutrient injection ports receive a high level of nutrients while others can be under starvation conditions.\(^{321}\) Cells circulating around a large scale bioreactor will therefore move through rapidly changing micro-environments\(^{167}\) and these cells respond very quickly to local changes in pH, dissolved oxygen and glucose concentration by rapid induction of different sets of genes.\(^{167,379}\) In large scale fermentors this can result in up to 25% less biomass compared to a laboratory scale fermenter.\(^{379}\) Xu et al.\(^{488}\) found that protein production was reduced by 12% when comparing a seven-litre laboratory fermentor with an industrial fermentor. Hewitt et al.\(^{167}\) found that although reduced mixing efficiency reduced the recombinant protein yield of the cells, it also increased the proportion of viable cells in the culture. Oxygen can also become limiting in large culture volumes, due to its low solubility. This can be overcome by increasing the aeration rate, using oxygen enriched air or pure oxygen, and limiting the growth rate.\(^{251}\) Pure oxygen is more expensive; however, Liu et al.\(^{271}\) found that sufficient dissolved oxygen could not be maintained with air alone. If sufficient oxygen is supplied it will not be a limiting factor for \textit{E. coli} growth.\(^{387}\) Acetate is produced by \textit{E. coli} growing under anaerobic or oxygen limiting conditions and in the presence of high levels of glucose. Acetate has been shown to reduce growth rate, biomass yield and maximum cell densities in high cell-density cultures when present at high concentrations\(^{153,194,278}\) and also to have a greater effect upon recombinant cells and those grown in defined media.\(^{227}\) The exact mechanism of this detrimental effect is unknown, but it has been suggested that acetate may suppress DNA and RNA synthesis and the production of proteins and lipids.\(^{82}\) Acetate production can be prevented by using glycerol as a carbon source, however glycerol is more expensive than glucose and cells grow more slowly.\(^{251}\) Reducing the temperature of the culture can be used to reduce nutrient uptake and growth, thus reducing the growth rate and the production of toxic by-products. It also reduces cellular oxygen demand.\(^{251}\) Acetate production can also be controlled by metabolic engineering. This can be done by preventing acetate production or increasing acetate consumption by the cells.\(^{387}\) Bauer et al.\(^{35}\) used a Pta\(^{-}\) mutant \textit{E. coli} strain to improve cell densities, while Farmer and Liao\(^{126}\) reduced acetate concentrations four-fold by overexpressing phosphoenolpyruvate carboxylase, which diverts carbon flux away from acetate production, and removing the \textit{fadR} gene, which encodes a repressor peptide that represses acetate metabolism in the presence of glucose. As these factors involved in scaling up fermentation can impact on protein yield, it is important to compare the results of the up-
scaled fermentations to the initial small scale cultures to ensure production efficiency is not lost.

1.11.3 Purifying recombinant protein: Affinity tags

Once a protein has been produced in *E. coli* it must then be purified. A frequent method is the use of affinity tags. Affinity tags are polypeptide fusion partners, which allow one step purification, have a minimal effect on tertiary structure and biological activity, and are easy to remove to leave the native protein. There are many types of affinity tags, the most common are small peptide tags which include the strep-tag, FLAG-tag, polyarginine-tag and his-tag. For some applications these small tags do not need to be removed, however, for most human therapeutic purposes or structural biology studies the tag should be removed to prevent any effect on biological function, immunogenicity or structure. Effects on tertiary structure and activity depend on the amino acid composition of the tag and its location. A disadvantage of small affinity tags for high throughput purification is they tend to be expensive and have relatively low binding capacities. The Strep-tag is a small eight-peptide tag that binds with high affinity to a streptavidin mutant (Strep-Tactin). Biotinylated proteins are also bound by Strep-Tactin, but this interaction can be blocked by avidin. The protein can be eluted under a variety of conditions and this method is therefore recommended for purifying metal containing proteins, active fusion proteins under anaerobic conditions, and for proteins to be used for NMR spectroscopy and crystallization. FLAG-tags consist of a hydrophobic eight-peptide fusion partner which can bind to the antibody M1. The proteins can be eluted by EDTA or lowering the pH, but the monoclonal antibody matrix is not as stable as that for other affinity tags (e.g. Strep-Tactin). However, FLAG-tags have been successfully used in a number of systems. Polyarginine-tags consist of four to five arginines and are used as a C-terminal tags which bind to cation exchange resin SP-Spandex and can be eluted by a NaCl gradient. His-tags are widely used and consist of several histidine residues. This system utilises immobilized metal affinity chromatography based on the interactions between a transition metal ion (e.g. Ni²⁺) immobilised on a nitrilotriacetic acid matrix and the histidine imidazole ring. Proteins containing consecutive histidines are bound to the metal ion and retained on the matrix. Other proteins containing histidine are washed off with low concentrations of free imidazole and the remaining bound proteins can then be eluted with higher imidazole concentrations. A disadvantage of imidazole is it can interfere with NMR experiments, competition experiments and can lead to protein aggregates.
tags can be placed on the N- or C-terminus depending on the protein and have been used successfully in both bacterial\textsuperscript{337,392} and eukaryotic\textsuperscript{23,355} expression systems. Lai et al.\textsuperscript{247} have successfully purified recombinant zoocin A using a his-tag.\textsuperscript{392} Affinity tags can be removed with a site specific protease and should not affect the protein activity.\textsuperscript{433} For example, factor Xa has been used to remove N-terminal tags. Cleavage can be carried out at temperatures from 4 to 25°C although ineffectual or non specific cleavage when using factor Xa has been reported.\textsuperscript{433,466} After cleavage the tags can be removed by binding to the immobilised matrix; however, the protease must be removed by chromatography.\textsuperscript{19}

1.12 Conclusions

The oral biofilm is a complex and organised microbial community containing many different bacterial species.\textsuperscript{2,231,244} Numerous studies have contributed to our growing understanding of the etiology of dental caries and the role of the mutans streptococci, particularly \textit{S. mutans}, in its development.\textsuperscript{53,294,447} These bacteria are both acidogenic and aciduric, rapidly producing acid from dietary carbohydrate.\textsuperscript{354} They are able to carry out this process in low pH environments, which results in demineralisation of the enamel, leading to decay.\textsuperscript{447,449} A range of broad-spectrum antimicrobials,\textsuperscript{148,162} including chlorhexidine\textsuperscript{348,408} and fluoride,\textsuperscript{217,291} have been shown to act against the oral biofilm, and other preventative measures, such as water fluoridation, have led to a significant decrease in the incidence of dental caries.\textsuperscript{178} However, dental caries continues to be a major problem.\textsuperscript{54} Many novel antimicrobials are being investigated as possible therapeutic agents for dental caries\textsuperscript{14,25,150,233} and several antimicrobial combinations have been found to be more effective than individual agents.\textsuperscript{100,101,236} Zoocin A is a D-alanyl-L-alanine endopeptidase\textsuperscript{138} that has activity against mutans streptococci, including \textit{S. mutans},\textsuperscript{188,390,392} and acts by cleaving the peptidoglycan cross-links.\textsuperscript{391} Monolaurin is a glycerol monoester of lauric acid that is particularly active against gram-positive bacteria.\textsuperscript{74,376} Possible antimicrobials are investigated using plaque models which aim to simulate the oral environment \textit{in vitro}. These models range from simple bacterial cultures to complex biofilm models which mimic the oral cavity environment.\textsuperscript{304,428} Simple models are inexpensive, easy to use, and utilised for preliminary studies, whereas complex models may give results that are more relevant to the \textit{in vivo} environment; although they are more expensive and require trained staff. It is therefore important to use a suitable model which meets the requirements of the individual study and experiments. To investigate the effectiveness of an antimicrobial protein, the protein must be produced at sufficient concentrations. This can be achieved using recombinant expression in
The expression system may need to be optimised, as final protein yield can be affected by the bacterial strain, the vector and inducer used, possible codon bias and the effects of up-scaling protein production.¹⁶,¹⁶⁷,²⁰⁵

1.13 Hypothesis and aims

This study hypothesised that zoocin A can be used to change the ecology of a plaque biofilm from a cariogenic to a non-cariogenic state. The aims of this study were: A) to produce zoocin A at sufficient concentrations to use as an antimicrobial in a biofilm model and to optimise the protein expression in a recombinant *E. coli* system; B) to develop a triple-species biofilm model suitable for investigating the effects of antimicrobials on an oral biofilm and C) to use the biofilm model to study the effects of zoocin A and lauricidin, both individually and in combination, upon a biofilm and to compare these results with the effects of known anti-plaque agents.
2. Materials and Methods

2.1 Stock materials and standard methods

2.1.1 Stock Solutions

All chemicals were obtained from Sigma Chemical Co., St Louis, MO, USA (Sigma), unless otherwise specified. All stock solutions were stored at room temperature unless otherwise stated and solutions that were sterilised by autoclaving were autoclaved at 121°C for 15 minutes unless otherwise described. Milli-Q deionised water (MQ water) used for the preparation of media and reagents was prepared using a Nanopure Diamond Life Science UV/UF Water System according to the manufacturer’s instructions (Barnstead International, Thermo Scientific, Waltham, MA, USA).

Filter sterilised stock solutions:
The following stock solutions were filter sterilised using a 0.22 µm pore size filter (Millipore Corporation, Bedford, MA, USA) and stored at -20°C until use.

Ampicillin
Ampicillin and MQ water were mixed to form a stock solution with a concentration of 100 mg/ml.

Arginine
Arginine and MQ water were mixed to form a stock solution with a concentration of 0.5 M.

Isopropyl-β-D-thiogalactoside
IPTG (Roche Molecular Biochemicals, Mannheim, Germany) and MQ water were mixed to form a stock solution with a concentration of 0.1 M.

Kanamycin
Kanamycin and MQ water were mixed to form a stock solution with a concentration of 25 mg/ml.
**Lysozyme**
Lysozyme and MQ water were mixed to form a stock solution with a concentration of 100 mg/ml.

**Menadione**
Menadione and MQ water were mixed to form a stock solution with a concentration of 5 mg/ml.

**Streptomycin**
Streptomycin and MQ water were mixed to form a stock solution with a concentration of 10 mg/ml.

**Urea**
Urea (Bio-Rad Laboratories, Hercules, CA, USA) and MQ water were mixed to form a stock solution with a concentration of 0.5 M.

**Other stock solutions:**

**Chloramphenicol**
Chloramphenicol and 95% ethanol (Ajax Finechem, Seven Hills, NSW, Australia) were mixed to form a stock solution with a concentration of 25 mg/ml. The solution was stored at 4°C.

**0.5M Ethylenediaminetetraacetic acid, pH 8.0**
Di-sodium EDTA (18.61 g) was mixed with reverse osmosis (RO) water and the pH adjusted to 8.0. The volume was made up to 100 ml with RO water and the solution autoclaved.

**Fluorescein isothiocyanate conjugated wheat germ agglutinin**
Fluorescein isothiocyanate conjugated wheat germ agglutinin (FITC-WGA) and MQ water were mixed to form a stock solution with a concentration of 1 mg/ml. The solution was stored at -20°C.

**Haemin**
Haemin was dissolved in 20 mM NaOH to a final concentration of 2.5 mg/ml. NaOH (Merck, Whitehouse Station, NJ, USA) (80 mg) was dissolved in 100 ml MQ. Haemin (125
mg) was mixed with 20 mM NaOH to a final volume of 50 ml. The solution was autoclaved and stored at 4°C.

2 M glucose
Dextrose (3.6 g) was dissolved in 10 ml RO water and autoclaved.

Glycerol (10%)
Glycerol (British Drug Houses Chemicals Ltd (BDH), Poole, Dorset, UK) (200 ml) was mixed with 1.8 L RO water and autoclaved.

Glycerol (20%)
Glycerol (BDH) (20 ml) was mixed with 80 ml RO water and autoclaved.

2 M Magnesium chloride
MgCl₂ (anhydrous, 1.9 g) was dissolved in 10 ml RO water and autoclaved.

Remazol brilliant blue solution
Remazol brilliant blue (1.88 g) and 1.5 g NaOH (Merck) were mixed with 150 ml RO water.

Rifampicin
Rifampicin and 95% methanol (Biolab, Scoresby, VIC, Australia) were mixed to form a stock solution with a concentration of 10 mg/ml. The solution was stored at -20°C.

0.35% Sodium chloride
NaCl (Scharlau Chemie, S.A., La Jota, Barcelona, Spain) (1.75 g) was dissolved in 500 ml RO water and autoclaved.

DNA gel electrophoresis:
50 x Tris-acetate ethylenediaminetetraacetic acid buffer, pH 8.0 (50 x TAE)
Tris base (Applichem gmbH, Darmstadt, Germany) (24.2 g) was dissolved in 60 ml of MQ water. Glacial acetic acid (5.71 ml) and 10 ml 0.5 M EDTA (pH 8.0) were added, and the volume made up to 100 ml with MQ water.
Agarose gel
Agarose (SeaKem LE agarose, Lonza, Basel, Switzerland) was added to 90 ml of 1 x TAE buffer to form a 1 or 1.5% solution. Gels were prepared as required.

Tracking dye
Bromophenol blue (0.025 g), 0.025 g xylene cyanol and 1.5 g ficoll 400 were dissolved in 10 ml RO water.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE):
30% acrylamide/bis solution
Acrylamide/N,N’-methylen-bis-acrylamide premix (Bio-Rad) (50 g) was dissolved in MQ water to a final volume of 100 ml. The solution was stored at 4°C for up to one month.

1.5 M Tris-HCl, pH 8.8
Tris base (Applichem) (18.17 g) was dissolved in MQ water. The pH was adjusted to 8.8 with 1 M HCl and the volume made up to 100 ml with MQ water. The solution was stored at 4°C.

0.5 M Tris-HCl, pH 6.8
Tris base (Applichem) (6.0 g) was dissolved in MQ water. The pH was adjusted to 6.8 with 1 M HCl and the volume made up to 100 ml with MQ water. The solution was stored at 4°C.

10% Sodium dodecyl sulphate
SDS (BDH) (10 g) was dissolved in MQ water to a final volume of 100 ml.

10% Ammonium persulphate
Ammonium persulphate (APS) (Bio-Rad) (100 mg) was dissolved in MQ water to a final volume of 1 ml. The solution was made fresh as required.

2 x SDS-PAGE sample buffer
SDS (BDH) (2.0 g), 0.5 g sucrose and 0.2 ml bromophenol blue were dissolved in 10 ml 0.5 M Tris-HCl (pH 6.8) and made up to a final volume of 50 ml with MQ water. Before each use 100 µl β-mercaptoethanol was added to 900 µl of the above solution.
Running buffer
Tris base (Applichem) (6.0 g), 28.8 g glycine and 2.0 g SDS (BDH) were dissolved in 2 L MQ water.

Itzhaki and Gill protein assay:
1% Copper sulfate
CuSO\(_4\).5H\(_2\)O (0.2 g) was dissolved in MQ water to a final volume of 20 ml.

30% Sodium hydroxide
NaOH (Merck) (6.0 g) was dissolved in MQ water to a final volume of 20 ml.

0.21% Copper sulfate in 30% sodium hydroxide
NaOH (Merck) (6.0 g) was dissolved in MQ water to a final volume of 16 ml and 4.2 ml of 1% CuSO\(_4\).5H\(_2\)O was added stepwise.

Expression and purification of recombinant protein:
Lysis buffer
Lysis buffer solution consisted of 50 mM NaH\(_2\)PO\(_4\) (BDH), 300 mM NaCl (Scharlau) and 10 mM imidazole dissolved in MQ water. The pH was adjusted to 8.0 and the solution was autoclaved.

Wash buffer
Wash buffer solution consisted of 50 mM NaH\(_2\)PO\(_4\) (BDH), 300 mM NaCl (Scharlau) and 20 mM imidazole dissolved in MQ water. The pH was adjusted to 8.0 and the solution was autoclaved.

Elution buffer
Elution buffer solution consisted of 50 mM NaH\(_2\)PO\(_4\) (BDH), 300 mM NaCl (Scharlau) and 250 mM imidazole dissolved in MQ water. The pH was adjusted to 8.0 and the solution was autoclaved.

Biofilm growth:
Chlorhexidine
Chlorhexidine gluconate (20% solution in water) was filter sterilised and the first 5 ml of filtrate discarded. The solution was stored in the dark at 4°C.
**Lauricidin**

Lauricidin (SEOLIM Corporation, Seoul, Korea) was mixed with 95% ethanol (Ajax Finechem) to form a stock solution with a concentration of 100 mg/ml. Lauricidin contains 90% monolaurin, 8% myristic acid and 2% capric acid. The solution was stored at -20°C.

**Phosphate buffered saline**

Phosphate buffered saline (PBS) consisted of 140 mM NaCl (Scharlau), 1 mM KH$_2$PO$_4$ (BDH), 10 mM Na$_2$HPO$_4$ and 3 mM KCl dissolved in RO water. The pH was adjusted to 7.2 and the solution was autoclaved.

**2.1.2 Media**

Commercial media were prepared according to the manufacturer’s specifications and sterilised by autoclaving: BHI broth, Todd Hewitt broth (THB), Columbia blood agar base (CAB), Luria broth (LB) and Luria broth agar (LBA), (Difco, Becton, Dickinson and Company (BD), Franklin Lakes, NJ, USA). CAB plates with antibiotics were prepared by supplementing CAB agar with 100 μg/ml rifampicin (CAB + rif) or 100 μg/ml streptomycin (CAB + strep) after autoclaving. LBA plates with antibiotics were prepared by supplementing LBA with: 100 μg/ml ampicillin (LBA + amp); 25 μg/ml kanamycin (LBA + kan); 34 μg/ml chloramphenicol (LBA + cm); 100 μg/ml ampicillin and 25 μg/ml kanamycin (LBA + amp + kan); or 100 μg/ml ampicillin, 25 μg/ml kanamycin and 34 μg/ml chloramphenicol (LBA + amp + kan + cm) after autoclaving. Blood agar (BA) was prepared by adding 5% (v/v) defibrinated sheep blood (New Zealand Venous Supplies, Tuakau, New Zealand) to CAB. Agar plates were stored at 4°C for a period of up to two weeks before use.

**Cadmium fluoride acriflavine tellurite agar**

Cadmium fluoride acriflavine tellurite (CFAT) agar was prepared as previously described by Zylber et al.$^{502}$ A 50 x CFAT stock was prepared by mixing 0.65 g cadmium sulphate, 4.25 g sodium fluoride, 0.6 g neutral acriflavine, 0.125 g potassium tellurite, 0.0625 g fuchsin with RO water to a total volume of 1 L. The stock was autoclaved and kept in the dark at 4°C. To make CFAT agar 15 g tryptic soy broth (TSB) (BD), 2.5 g dextrose and 7.5 g agar (BD) were mixed with RO water to a final volume of 470 ml and autoclaved. After cooling to 50°C, 25 ml of defibrinated sheep blood (NZ Venous Supplies) and 5 ml of 50 x CFAT
solutions were added to the agar. Agar plates were stored at 4°C for a period of up to two weeks before use.

*Basal medium mucin*

Basal medium mucin (BMM) was prepared as previously described by Wong and Sissons. For each litre of BMM, 2.5 g of Type III partially purified porcine stomach mucin was dissolved in 100 ml RO water. The mixture was heated at 80°C for one hour then cooled and stored overnight at 4°C before being filtered through No. 2 and then No. 1 filter paper (Whatman Ltd., Maidstone, UK) using a vacuum pump. The filtered mucin and 2.5 ml of a 2.5 mg/ml haemin stock were added to a KCl solution (trypticase peptone (5 g) (BD), 10 g proteose peptone (BD), 5 g yeast extract (BD) and 2.5 g KCl mixed with 500 ml RO water) and RO water was added to give a final volume of 995 ml. The solution was autoclaved and allowed to cool before the addition of 0.2 ml of 5 mg/ml menadione, 2 ml of 500 mM urea and 2 ml of 500 ml arginine. The BMM was stored at 4°C.

*1/5 strength brain heart infusion broth with 1% sucrose*

BHI (BD) (14.8 g) was mixed with RO water to a final volume of 1.5 L. Sucrose (20 g) was mixed with RO water to a final volume of 500 ml. The solutions were autoclaved separately and then mixed after cooling to room temperature.

*1/3 strength brain heart infusion broth with 1% sucrose*

BHI (BD) (24.6 g) was mixed with RO water to a final volume of 1.5 L. Sucrose (20 g) was mixed with RO water to a final volume of 500 ml. The solutions were autoclaved separately and then mixed after cooling to room temperature.

*1/5 strength Todd Hewitt broth with 1% sucrose*

THB (BD) (12 g) was mixed with RO water to a final volume of 1.5 L. Sucrose (20 g) was mixed with RO water to a final volume of 500 ml. The solutions were autoclaved separately and then mixed after cooling to room temperature.

*1/3 strength Todd Hewitt broth with 1% sucrose*

THB (BD) (20 g) was mixed with RO water to a final volume of 1.5 L. Sucrose (20 g) was mixed with RO water to a final volume of 500 ml. The solutions were autoclaved separately and then mixed after cooling to room temperature.
Super optimal broth

Super optimal broth (SOB) consisted of 10 g bacto-trypotne (BD), 2.5 g yeast extract (BD) and 0.25 g NaCl (Scharlau) dissolved in 400 ml RO water. Five millilitres of 250 mM KCl was added to the solution, the total volume made up to 500 ml with RO water and the solution was autoclaved.

Super optimal broth with catabolite repression

SOB with catabolite repression (SOC) consisted of 9.8 ml sterile SOB supplemented with 100 μl of 2 M MgCl₂ and 100 μl of 2 M glucose.

2.1.3 Bacterial strains

Bacterial strains used in this study are listed in Table 2.1. Stock cultures of all strain were stored in 20% glycerol at -70°C. Strains not in regular use were subcultured from freezer stock cultures as required. Strains in regular use were subcultured onto agar plates and stored at 4°C for up to one week. E. coli strains were maintained on LBA supplemented with the appropriate antibiotics and incubated at 37°C for 24 hours. E. coli broth cultures were prepared in LB with the appropriate antibiotics and incubated at 37°C with shaking at 200 rpm on an incubator shaker (Innova 44, New Brunswick Scientific (NBS), Edison, New Jersey) for 18 hours. Streptococcal and actinomyces species were maintained on BA incubated at 37°C in air supplemented with 5% CO₂. S. pyogenes FF22 and S. equi subspecies zooepidemicus 4881 were incubated for 24 hours and broth cultures were prepared in THB, while other streptococcal and actinomyces species were incubated for 48 hours and broth cultures were prepared in BHI. Both THB and BHI overnight cultures were incubated at 37°C in air supplemented with 5% CO₂ for 18 hours.

2.1.4 Electrophoresis procedures for DNA

Agarose gels (1 or 1.5%) were subject to electrophoresis in 1 x TAE buffer at a constant voltage of 80 V (Power Pac 300, Bio-Rad), in a submarine gel tank (Owl, Separation Systems, Portsmouth, NH, USA). DNA (8 μl sample) was mixed with 2 μl tracking dye and 8 μl loaded into the gel. A DNA marker (1 kb plus DNA ladder or 100 bp DNA ladder, Invitrogen Life Technologies, CA, USA) was used to allow estimation of the molecular weight. Electrophoresis was stopped when the tracking dye had migrated two thirds of the way down the gel.
<table>
<thead>
<tr>
<th>Bacterial species and strain</th>
<th>Plasmid</th>
<th>Description</th>
<th>Selection system</th>
<th>Source or reference</th>
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<tr>
<td><em>Streptococcus mutans</em> 10449</td>
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<td>Rifampicin resistance</td>
<td>100 µg/ml rifampicin</td>
<td>DMCC</td>
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<tr>
<td><em>Streptococcus oralis</em> 34</td>
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<td>Streptomycin resistance</td>
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<td>DMCC</td>
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<tr>
<td><em>Actinomyces viscosus</em> T14AV</td>
<td></td>
<td></td>
<td>CFAT agar</td>
<td>DMCC</td>
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<tr>
<td><em>Streptococcus equi</em> subspecies</td>
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<td>Zoocin A producer strain</td>
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<td>DMCC</td>
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<tr>
<td><em>zooepidemicus</em> 4881</td>
<td></td>
<td></td>
<td></td>
<td>DMCC</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em> FF22</td>
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<td></td>
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<td>DMCC</td>
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<tr>
<td><em>Escherichia coli</em> DH5α</td>
<td>pQE-80L ZooA1</td>
<td></td>
<td>100 µg/ml ampicillin</td>
<td>DMCC</td>
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<tr>
<td><em>Escherichia coli</em> M15</td>
<td>pREP4</td>
<td></td>
<td>25 µg/ml kanamycin</td>
<td>DMCC</td>
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Table 2.1: continued

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<tr>
<th>Bacterial species and strain</th>
<th>Plasmid</th>
<th>Description</th>
<th>Selection system</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> M15</td>
<td>pQE-80L ZooA1, pREP4</td>
<td>Recombinant zoocin A producer strain</td>
<td>100 µg/ml ampicillin plus 25 µg/ml kanamycin</td>
<td>Dr Robin Simmonds(^{247})</td>
</tr>
<tr>
<td><em>Escherichia coli</em> BL21</td>
<td><em>CodonPlus-(DE3)-RIL</em></td>
<td><em>CodonPlus</em> strain</td>
<td>34 µg/ml chloramphenicol</td>
<td>Stratagene</td>
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</tbody>
</table>

*DMCC = Department of Microbiology and Immunology Culture Collection, University of Otago, New Zealand.*

*Stratagene = Stratagene, Agilent Technologies, Santa Clara, CA, USA.*
Gels were then stained in 0.5 µg/ml ethidium bromide for 30 minutes and destained in RO water for 15 minutes. Bands were viewed under ultraviolet light and photographed using a Gel DOC 2000 gel documentation system (Bio-Rad) and printed on a Mitsubishi video graphic printer.

2.1.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

A 7.5% resolving gel was prepared by mixing 2.5 ml 30% acrylamide/bis solution, 2.5 ml 0.5 M Tris-HCl, pH 8.8; 100 µl 10% SDS, and 4.85 ml MQ water. Freshly prepared 10% APS (50 µl) and 5 µl of N,N,N’,N’-tetramethylethylenediamine (TEMED) was then added. The gel mix was poured into a Mini-PROTEAN II multicasting chamber (Bio-Rad), overlaid with MQ water (to exclude air) and left to set for 30 minutes. The overlaying MQ water was removed prior to pouring the stacking gel. A 4% stacking gel was prepared by mixing 330 µl 30% acrylamide/bis solution; 630 µl 0.5 M Tris-HCl, pH 6.8; 25 µl of 10% SDS; and 1.5 ml MQ water. TEMED (2.5 µl) and 12.5 µl 10% APS were then added. The stacking gel was poured, a comb inserted and the gel allowed to polymerise before storage at 4ºC for one hour.

Protein preparations (10 µl) were mixed with an equal volume of 2 x SDS-PAGE sample buffer, and boiled for two minutes before loading 10 µl onto the gel. Five microlitres of a low molecular weight protein standard (Bio-Rad) was loaded onto the gel for size determination of the protein.

Electrophoresis was performed at a constant voltage of 100 V (Model 250 Power Supply, Bethesda Research Laboratories, Life Technologies Inc., Gaithersburg, MD, USA) in a Mini-PROTEAN II electrophoresis cell (Bio-Rad) containing running buffer. Electrophoresis was stopped when the bromophenol blue dye had migrated to the bottom of the gel. The gel was removed from between the glass plates, rinsed briefly in RO water, and immersed in Coomassie Blue stain solution (Bio-Rad) for 2.5 hours. The gel was destained overnight in RO water. Bands were viewed under white light, photographed using a Gel DOC 2000 gel documentation system (Bio-Rad) and printed on a Mitsubishi video graphic printer.

2.1.6 Preparation of electrocompetent cells

SOB (two flasks of 500 ml) was inoculated with 5 ml of overnight culture of the desired bacterial strain. Cultures were incubated at 37°C with shaking at 200 rpm in an incubator
shaker (Innova 44, NBS) until the OD at 595 nm (OD\textsubscript{595}) reached 0.7. The culture was then centrifuged at 7520 x \textit{g} for 10 minutes at 4°C. The supernatant was discarded and the cells resuspended in 200 ml cold 10% glycerol. The centrifugation was repeated, the supernatant discarded, and the cells resuspended in 150 ml cold 10% glycerol. The centrifugation was performed a third time, the supernatant discarded, and the cells resuspended in 10 ml cold 10% glycerol. After a fourth centrifugation the cells were resuspended in 5 ml cold 10% glycerol. The electrocompetent cells were divided into 100 μl aliquots and snap frozen in a dry ice and ethanol bath. The cells were promptly stored at -70°C. Before experimental use the cells were tested for electrocompetence using test plasmid pFX3 with erythromycin resistance (50 μg/ml).

\textbf{2.1.7 Electroporation}

Electroporation was performed using an \textit{E. coli} TransPorter (Biotechnologies and Experimental Research inc., San Diego, USA), a Pharmacia LKB 2197 power supply (Pharmacia LKB, Broma, Sweden), and 0.1 cm electrode gap Gene Pulser cuvettes (Bio-Rad). Electrocompetent cells were thawed on ice for 10 minutes and Gene Pulser cuvettes (0.1 cm, Bio-Rad) were chilled on ice for 10 minutes before use. DNA (0.1–1 ng) was then added to 40 μl of electrocompetent cells and incubated on ice for 10 minutes. Cells were electroporated for one second with 200 ohms at 1.5 kV (25 μF) of electricity. Following electroporation 950 μl of SOC was added to the cells and they were transferred to a 25 ml screw-capped glass vial, which was incubated at 37°C in an incubator shaker (Innova 4000 Incubator shaker, NBS) at 200 rpm for one hour. After incubation serial 10-fold dilutions were prepared in SOC and 100 μl volumes were spread plated onto LBA supplemented with the appropriate antibiotics. The plates were incubated at 37°C overnight. Electrocompetent cells were also electroporated with MQ water as a control.

\textbf{2.2 Production and quantification of zoocin A}

\textbf{2.2.1 Small scale zoocin A production}

Recombinant zoocin A was produced as previously described by Lai et al.\textsuperscript{247} using the recombinant zoocin A producer strain \textit{E. coli} M15 pREP4, pQE-80L ZooA1. A 25 ml overnight culture of \textit{E. coli} M15 pREP4, pQE-80L ZooA1 was used to inoculate 500 ml of LB with 1% glucose, 100 μl/ml ampicillin and 25 μg/ml kanamycin and the culture was
incubated at 37°C with shaking at 200 rpm in an incubator shaker (Innova 44, NBS) until the OD<sub>595</sub> was 0.7 (Novaspec II, Biochrom, Cambridge, UK). IPTG (0.5 ml of a 0.1 M stock) was added and the culture incubated at 37°C with shaking at 200 rpm in an incubator shaker (Innova 44, NBS) for a further four hours. The cells were then harvested by centrifugation at 3840 x g for 15 minutes at 4°C, and the bacterial pellet resuspended in 30 ml lysis buffer and stored at -20°C overnight. The suspended pellet was thawed, lysozyme was added to a final concentration of 1 mg/ml and the solution incubated on ice for 30 minutes. The lysate was then centrifuged for 20 minutes at 15,300 x g at 4°C and the supernatant collected. The zoocin A in the supernatant was purified using Ni-NTA affinity purification as described below in section 2.2.3.

2.2.2 Large scale zoocin A production in benchtop fermentor

A 10 L culture of <i>E. coli</i> M15 pREP4, pQE-80L ZooA1 was grown using the BioFlo410 fermentor (NBS). Four litres of 2.5 x strength LB was transferred to the fermentor along with 4.88 L RO water and 1 ml of antifoam 204 that had been mixed with 99 ml cold RO water. The temperature was set to 37°C with an agitation speed of 200 rpm. The media was sterilised at 121°C for 45 minutes. Following sterilisation the media was allowed to cool to 37°C. Glucose (100 g) was dissolved in RO water to a final volume of 750 ml and autoclaved separately. A 25 ml overnight culture of <i>E. coli</i> M15 pREP4, pQE-80L ZooA1 was used to inoculate a 250 ml LB starter culture (supplemented with 100 μl/ml ampicillin and 25 μg/ml kanamycin) of <i>E. coli</i> M15 pREP4, pQE-80L ZooA1 which was incubated overnight at 37°C with shaking at 200 rpm in an incubator shaker (Innova 44, NBS). The sterile media in the fermentor was supplemented with the 750 ml glucose solution, 10 ml of 100 mg/ml ampicillin and 10 ml of 25 mg/ml kanamycin. A blank sample was taken using the sampling port and the starter culture was added. Compressed air was sparged through the culture at a rate of 10 standard litres per minute. The OD<sub>595</sub> was monitored by taking samples through the sampling port. When the OD<sub>595</sub> reached 0.7, 10 ml of 0.1 M IPTG was added. The culture was incubated for a further four hours, then the culture was drained from the fermentor and centrifuged at 4680 x g for 15 minutes at 5°C. The cells were resuspended in 300 ml lysis buffer and stored at -20°C until frozen. The resuspended pellet was thawed, lysozyme added to a final concentration of 1 mg/ml, and the solution incubated on ice for 30 minutes. The lysate was then centrifuged at 15,300 x g for 20 minutes at 4°C and the supernatant collected. The zoocin A in the supernatant was then purified using Ni-NTA affinity purification as described below in section 2.2.3.
2.2.3 Purification of zoocin A

Zoocin A was purified using Ni-NTA affinity purification (Qiagen, Hamburg, Germany). A 500 μl sample of the supernatant was retained and the remainder was loaded onto an equilibrated agarose/nickel column containing Ni-NTA beads (Qiagen). The column was 30 mm in diameter. For small scale zoocin A production it contained 30 mm of beads and for large scale production it contained 110 mm of beads. The supernatant was allowed to slowly pass through the column and the eluate collected. Wash buffer (half the volume of the supernatant) was loaded on to the column, run through and collected in two equal volumes. The zoocin A protein was then eluted from the nickel column using elution buffer. For small scale zoocin A production 60 ml of elution buffer was added to the column and collected in six 10 ml volumes. For large scale zoocin A production 300 ml of elution buffer was loaded onto the column and collected in six 50 ml volumes. SDS-PAGE was performed on each of the eluate fractions to determine if zoocin A was present. A spot test was performed to give an estimate of zoocin A activity in each fraction and the fractions containing high levels of zoocin A were pooled. The protein concentration was tested by Itzhaki assay and protein activity tested using the spot test.

2.2.4 Estimation of recombinant protein activity by the spot test

The spot test was similar to the method described by Simmonds et al. Zoocin A samples were serially diluted two-fold 12 times in lysis buffer. Twenty microlitres of each dilution was spotted onto a CAB agar plate and spots were allowed to absorb. A lawn of S. pyogenes FF22 was swabbed onto each plate and plates were incubated at 37°C in air supplemented with 5% CO₂ for 18 hours. The inverse of the final dilution of zoocin A that formed a zone of inhibition was recorded as the zoocin A titre in AU/ml.

2.2.5 Dialysis and lyophilisation of zoocin A to produce a zoocin A standard

Fifteen centimetres of dialysis tubing that retains proteins with a molecular weight above 12,000 Da and a capacity of 60 ml per 30.5 cm (Sigma) was soaked in RO water for four hours with gentle heating, then rinsed thoroughly to remove glycerol from the tubing. The first elution fraction (10 ml) from a small scale zoocin A production and purification run was tested for zoocin A activity with the spot test and then dialysed in 5 L of RO water with
stirring for 36 hours with the dialysing water changed every 12 hours. The zoocin A (9.5 ml) was then removed from the dialysis tubing and filter sterilised. A 500 µl aliquot was removed for zoocin A titre calculation by spot test and the remaining zoocin A was stored at -20°C for lyophilisation. The zoocin A sample was lyophilised in a FreeZone 6 console freeze dry system (LABCONCO, Kansas City, MO, USA). The lyophilised sample was then weighed, and resuspended in MQ water to give a final concentration of 5 mg/ml. The activity of the resuspended zoocin A was also calculated by spot test.

**2.2.6 Estimation of recombinant protein concentration by Bradford assay**

Bovine serum albumin (BSA) protein standards were prepared in MQ water. BSA (1 mg/ml) was diluted in MQ water to 15.62 µg/ml and this sample was serially diluted two-fold to 0.98 µg/ml. The zoocin A sample was diluted in MQ water to obtain a reading within the linear range of the assay. Each BSA and zoocin A dilution was transferred to a 96-well flat bottom clear polystyrene microtitre plate (BD Falcon, BD) in three 160 µl aliquots. MQ water was used as a blank. Forty microlitres of Bio-Rad Protein Assay Reagent (Bio-Rad) was added. The microtitre plate was incubated at room temperature for 10 minutes and the OD 595 of the samples recorded in an Infinite M200 plate reader (Tecan Group Ltd., Männedorf, Switzerland). The BSA samples were used to make a standard curve and the amount of zoocin A present in the sample was calculated by comparison to that curve.

**2.2.7 Estimation of recombinant protein concentration by Itzhaki assay**

The protein assay was carried out according to the methods described by Itzhaki and Gill et al.\(^{186}\) Protein standards were prepared from either BSA (1 mg/ml) or zoocin A (5 mg/ml). Two sets of protein standards were prepared in 1.5 ml microcentrifuge tubes. For each set the protein standard was diluted in MQ water to final concentrations of 200, 180, 120, 80, 40 and 0 µg/ml with a total volume of 1 ml each. Two sets of the unknown zoocin A protein sample were prepared. For each set the protein samples were diluted in MQ water 1:10, 1:50 and 1:100 to a total volume of 1 ml. To one set of standards and samples, 0.5 ml of 0.21% CuSO\(_4\).5H\(_2\)O in 30% NaOH was added. To the second set 0.5 ml of 30% NaOH was added. The tubes were then mixed and left at room temperature for 10 minutes. Using the 0 µg/ml sample with 0.21% CuSO\(_4\).5H\(_2\)O as a blank, the absorbance of all standards and samples with 0.21% CuSO\(_4\).5H\(_2\)O added were read at 340 nm. Using the 0 µg/ml sample with 30% NaOH as a blank, the absorbance of all standards and samples with 30% NaOH added were read at
340 nm. The differences between the two standards and samples were then calculated and used to make a standard curve, and the amount of unknown protein for the protein samples determined.

2.2.8 Dye release assay (DRA)

2.2.8.1 Production of dye labelled substrate (DLS)
A 10 ml overnight culture of *S. pyogenes* FF22 was used to inoculate 500 ml of pre-warmed THB which was incubated at 37°C in air supplemented with 5% CO₂ for 18 hours. The culture was centrifuged at 15,300 x g for 20 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 200 ml of 0.35% NaCl and centrifuged again as above. The supernatant was discarded and the pellet resuspended in 100 ml MQ water and centrifuged as above. The pellet was resuspended in 30 ml remazol brilliant blue solution per 0.6 g of cells (wet weight) and transferred to a 250 ml conical flask. The cells were incubated at 37°C with shaking at 200 rpm in an incubator shaker (Innova 44, NBS) for six hours, then incubated at 4°C on a platform shaker (Excella E10, NBS) at 200 rpm for a further 18 hours. Following incubation cells were centrifuged at 15,300 x g for 20 minutes at 4°C. The supernatant was discarded and cells resuspended in 225 ml MQ water. This centrifugation step was repeated until the supernatant was free of blue dye (five washes). Finally the cell pellet was resuspended in 20 ml MQ water and 1 ml aliquots of DLS were stored at -20°C. Before use in the DRA, DLS was thawed and centrifuged for five minutes at 13,000 x g. The supernatant was discarded and the pellet resuspended in 50 mM Tris-HCl, pH 8.0. This wash step was repeated until no blue dye was present in the supernatant. A sample of DLS was diluted two-fold to 1:32 and the OD₅₉₅ of a 100 µl sample was measured in an Infinite M200 plate reader (Tecan Group Ltd.). The remaining DLS was then diluted to the required OD₅₉₅ for use in the DRA.

2.2.8.2 Developing a standard curve for the dye release assay
The DRA was carried out as previously described by Tran. Zoocin A was diluted in 50 mM Tris-HCl, pH 8, to 100 AU/ml then serially diluted two-fold to 0.78 AU/ml. DLS (100 µl) with an OD₅₉₅ of 1.5 was mixed with 100 µl of each zoocin A dilution and incubated for 30 minutes. Reactions were halted by the addition of 100 µl of 95% ethanol (Ajax Finechem) and brief vortexing. Samples were centrifuged for five minutes at 13,000 x g and the OD₅₉₅ of 100 µl of the supernatant was measured.
This experiment was repeated with zoocin A concentrations of 10, 7.5, 5 and 2.5 AU/ml, with reaction incubation times of 10, 20 and 30 minutes and with DLS concentrations of 1.0, 1.5, 2.0 and 3.0, with an incubation time of 10 minutes.

**2.2.8.3 Dye release assay**

Zoocin A standards were prepared by diluting zoocin A to concentrations of 10, 7.5, 5 and 2.5 AU/ml. MQ water was used as a blank. The concentration of the unknown zoocin A sample was estimated using the spot test and serial dilutions prepared to include a sample in the range of the standard curve. DLS (100 µl) with an OD$_{595}$ of 2.0 was mixed with 100 µl of each zoocin A dilution (standards and samples) and incubated for 10 minutes. Reactions were halted by the addition of 100 µl of 95% ethanol (Ajax Finechem) and briefly vortexing. Samples were centrifuged for five minutes at 13,000 x g and the OD$_{595}$ of 100 µl of the supernatant was measured.

**2.2.9 Zoocin A production by the original producer strain and recombinant E. coli**

**2.2.9.1 Zoocin A production by S. equi subspecies zooepidemicus**

A 25 ml overnight culture of *S. equi* subspecies *zooepidemicus* 4881 was used to inoculate a 500 ml flask of THB which was incubated at 37°C in air supplemented with 5% CO$_2$. The OD$_{595}$ was recorded every hour. The culture was centrifuged at 3840 x g for 15 minutes at 5°C, four hours after an OD$_{595}$ of 0.7 had been reached. The supernatant was retained and tested for zoocin A activity with the DRA.

**2.2.9.2 Zoocin A production by E. coli M15, pQE-80L ZooA1, pREP4**

Zoocin A was produced using the small scale zoocin A production methods described in section 2.2.1, except the OD$_{595}$ was recorded every hour. The culture was then centrifuged, the pellet lysed and centrifuged using the methods above. The lysate supernatant was retained and the zoocin A activity was tested using the spot test and DRA.

This experiment was repeated and following induction of the culture with IPTG 25 ml samples of culture were taken at the time of induction and every hour following, for five hours. Each sample was centrifuged at 3840 x g for 15 minutes at 5°C and the pellet was retained, resuspended in 2.5 ml of lysis buffer and stored at -20°C overnight. Samples were then thawed, lysed and centrifuged as described in section 2.2.3. The supernatant was tested for zoocin A production with the spot test and DRA.
2.2.10 Zoocin A production with varied IPTG concentrations

Four flasks of 125 ml LB supplemented with 1% glucose, 100 µg/ml ampicillin and 25 µg/ml kanamycin were inoculated with 6.25 ml overnight culture of *E. coli* M15 pREP4, pQE-80L ZooA1. The cultures were incubated at 37°C with shaking at 200 rpm in an incubator shaker (Innova 44, NBS). The OD was monitored and when they reached an OD<sub>595</sub> of 0.7 a 10 ml sample was taken and cultures were induced by addition of IPTG to attain a final concentration of 0.01, 0.1, 0.5 or 1 mM. Following induction, cultures were incubated at 37°C with shaking at 200 rpm in an incubator shaker (Innova 44, NBS). A 10 ml sample was taken every hour and the OD<sub>595</sub> recorded. Each sample was centrifuged at 2860 x g for 15 minutes at 5°C and the pellets resuspended in 1 ml lysis buffer and stored at -20°C overnight. Cells were then thawed, lysed and centrifuged as described in section 2.2.3. The supernatant was tested for zoocin A activity with the spot test.

2.2.11 Analysis of zoocin A gene for rare codons

The zoocin A sequence on plasmid pQE-80L ZooA1 (GenBank accession number U50357) was examined for codons that rarely occur in *E. coli* (a codon was considered rare if it is present in less than 10% of occurrences for a particular amino acid).

2.2.12 Site-directed mutagenesis of first AGA codon in zoocin A gene

2.2.12.1 Site-directed mutagenesis primer design

Site-directed mutagenesis was used to change the first AGA codon present in the zoocin A gene in pQE-80L ZooA1 to CGT. Site-directed mutagenesis primers were designed to be 25 to 45 bp long, with the mismatch in the middle of the primer and with 10 to 15 base pairs of matching sequence on each side. Primers were required to have a GC content of more than 40%, terminate in at least one G or C base and have a melting temperature (T<sub>m</sub>) of more than 78°C using the formulae T<sub>m</sub> = 81.5 + 0.41(%GC) - 675/N - %mismatch (where N is the primer length in base pairs, %GC is the percentage of bases that are G or C, %mismatch is the percentage of bases that do not match the target sequence and %GC and %mismatch are whole numbers). Primers were high-performance liquid chromatography (HPLC) purified and are listed in Table 2.2.
Table 2.2: Primers used in this study

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<td>GGACTAHAAGGGTATCTAAT</td>
<td>Weisburg et al., 1991⁴⁷⁰</td>
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<tr>
<td>16S rev</td>
<td>AGAGTTTGATCMTGG</td>
<td>Weisburg et al., 1991⁴⁷⁰</td>
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</table>
| fwd 102 ZooA AGA1 mut | 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2.2.12.2 Amplification of pQE-80L with site-directed mutagenesis primers

The plasmid pQE-80L ZooA1 was purified from E. coli M15, pQE-80L ZooA1, pREP4 using a miniprep kit (Invitrogen). Site-directed mutagenesis primers (Table 2.2) were used in a PCR to amplify the pQE-80L plasmid. All reagents for the PCR were obtained from Novagen (Novagen, Darmstadt, Germany). A reaction mix consisted of 27.3 μl MQ water, 5 μl of 10 x KOD buffer, 5 μl 2 mM dNTPs, 2 μl 25 mM MgSO₄, 4.2 μl forward primer and 4.5 μl reverse primer (final concentrations of 0.3 μM), 1 μl of template DNA and 1 μl of KOD polymerase. Amplification was performed in a thermocycler (Mastercycler, Eppendorf, Hamburg, Germany) using the following parameters: Cycle 1, denaturation temperature of 95°C for two minutes; cycles 2–17, denaturation temperature of 94°C for 15 seconds; annealing temperature of 55°C for 30 seconds, and extension temperature of 68°C for five minutes. The PCR product was visualised on a 1% agarose gel.

2.2.12.3 Digestion, ligation and electroporation of site-directed mutagenesis PCR product

The original template DNA in the PCR product was digested by DpnI by the addition of 5 μl Biolabs buffer 1 (Biolabs, Ipswich, MA, USA) and 1 μl DpnI (Biolabs) to the PCR reaction mix and incubation of the reaction at 37°C for one hour. The product was then purified using a PCR purification kit (Axygen, Union City, CA, USA) according to the manufacturer’s instructions. The PCR product was ligated using DNA ligase. Each reaction mix contained 2 μl 10 x buffer, 2.5 μl 40% polyethylene glycol (PEG, MW 8000), 14.5 μl PCR product, 1 μl DNA ligase (Biolabs) and was incubated at 16°C for 18 hours. The reaction was then halted by incubating at 65°C for 10 minutes. DNA was purified using Novagen pellet paint co-precipitant according to the manufacturer’s instructions (Novagen, Merck KGaA, Darmstadt, Germany). The purified mutated plasmid was then transformed into E. coli DH5α by electroporation. Transformed cells were spread on LBA + amp and incubated at 37°C for 18 hours.

2.2.12.4 Colony PCR of transformed cells

Twenty transformed PCR colonies were used in a colony PCR to check for the zoocin A gene insert. Primers were designed to target the pQE-80L plasmid and amplify the inserted zoocin A gene and are listed in Table 2.2. Primers were designed to be 18 to 22 bp long and had a GC content between 40 and 60%, terminated in at least one G or C base and had a Tₘ between 52 and 58°C. A reaction mix consisted of 34.5 μl MQ water; 5 μl 10 x KOD buffer; 5 μl 2 mM dNTPs; 1.5 μl forward primer and 1.5 μl reverse primer (final concentrations of 0.3 μM);
and 1 μl KOD polymerase. Template DNA was added by plucking a colony with a sterile toothpick and dipping it in the reaction mix prior to the addition of the KOD polymerase. Toothpicks were then struck on LBA + amp and incubated at 37°C for 18 hours. Amplification was performed with a thermocycler (Mastercycler, Eppendorf) using the following parameters: Cycle 1, denaturation temperature of 95°C for two minutes; cycle 2, 94°C for two minutes; cycles 3–32, denaturation temperature of 94°C for 15 seconds; annealing temperature of 48°C for 30 seconds, and extension temperature of 72°C for 45 seconds; cycle 33, denaturation temperature 94°C for 30 seconds, annealing temperature of 48°C for 30 seconds and elongation temperature of 72°C for 1 minute. PCR products were visualised on a 1% agarose gel. The mutated plasmid was purified from four colonies containing the zoocin A gene using a miniprep kit (Invitrogen). The amount of product was determined using a Nanodrop spectrophotometer (Nanodrop Technologies, Long Island, NY, USA). The zoocin A gene was then sequenced by the Massey DNA Sequencing Facility (Allen Wilson Centre, Massey University, Palmerston North, New Zealand) and sequencing reactions were prepared according to their instructions. The resulting sequence was analysed using SeqED software (DNASTAR Incorporated). Sequences were aligned and the change of the first AGA codon to CGT was checked.

2.2.12.5 Transformation of E. coli pREP4 with mutated plasmid

The mutated plasmid was purified using a miniprep kit (Invitrogen) and the amount of DNA determined using a Nanodrop spectrophotometer (Nanodrop Technologies). The plasmid was inserted into E. coli pREP4 by electroporation and transformed cells were spread on LBA + amp + kan and incubated at 37°C for 18 hours. Twelve transformed colonies were used in a colony PCR to check for the zoocin A gene as described above. Ten colonies containing the pQE-80L plasmid with the zoocin A gene were screened for altered zoocin A production (transformants 1 to 10).

2.2.13 Transformation of E. coli BL21-CodonPlus-(DE3)-RIL with pQE-80L ZooA1 and pREP4

E. coli BL21-CodonPlus-(DE3)-RIL competent cells (Stratagene, Agilent Technologies, Santa Clara, CA, USA) were transformed with pQE-80L ZooA1 and pREP4 according to the manufacturer’s instructions. To confirm the presence of the three plasmids, plasmids were purified from each of the six transformed colonies, and E. coli BL21-CodonPlus-(DE3)-RIL, E. coli M15, pREP4, and E. coli DH5α, pQE-80L ZooA1 using a miniprep kit (Invitrogen).
These plasmids were digested with Hind III and Bgl II, individually and in combination. All reagents were supplied by New England Biolabs (Ipswich, MA, USA). Reaction mixes consisted of 1 μg plasmid DNA, 5 μl 10 x buffer 3, 0.5 μl Hind III, 1.5 μl Bgl II and MQ water to a total volume of 50 μl. Reactions were incubated at 37°C for one hour and plasmid fragments were then visualised by running 5 μl of the reactions on a 1% agarose gel. Six transformed colonies were screened for altered zoocin A production (transformants A to F).

2.2.14 Screening for altered zoocin A production of transformed cells

Transformants 1 to 10, A to F, E. coli M15, pQE-80L, pREP4, and E. coli M15 pREP4 were tested for zoocin A production. Cultures were grown in flasks of 125 ml LB supplemented with 1% glucose and the appropriate antibiotics. Flasks were inoculated with 6.25 ml overnight culture of the strain to be tested. The cultures were incubated at 37°C with shaking at 200 rpm in an incubator shaker (Innova 44, NBS). The OD of the cultures was monitored and when they reached an OD595 of 0.7 a 10 ml sample was taken and cultures were induced with 0.1 mM IPTG. Following induction cultures were incubated at 37°C with shaking at 200 rpm in an incubator shaker (Innova 44, NBS). A 10 ml sample was taken every hour and the OD595 recorded. Each sample was centrifuged at 2860 x g for 15 minutes at 5°C. The pellets were resuspended in 1 ml lysis buffer and stored at -20°C overnight. Cells were then thawed, lysed and centrifuged as described in section 2.2.3 above. The supernatant was tested for zoocin A production with the spot test.

2.3 Biofilm model development

2.3.1 Description of biofilm apparatus

The biofilm apparatus consisted of a large circular brass block (150 mm in diameter and 73 mm high) containing nine internal chambers 35 mm high and 18 mm in diameter. The top of the block was removable to allow access to the internal chambers, as shown in Figure 2.1 A. Each internal chamber was surrounded by an O-ring to create a seal. Each chamber housed a small glass cup (35 mm high, with a 12 mm internal diameter (ID)) with a coarsely sintered glass bottom. Each cup held 1 g of glass beads, 1 mm in diameter, as shown in Figure 2.1 B. These glass beads provided a surface for the biofilm to grow upon. Nutrients were housed in three 2 L bottles, each of which was connected to three of the biofilm chambers by tubing.
Figure 2.1: Biofilm apparatus components. A: Biofilm apparatus, open to display nine internal chambers; B: Glass cup with sintered glass bottom, containing glass beads 1 mm in diameter; C: Attachment of tubing to 2 L nutrient feed bottle; D: Division and reduction of tubing connecting the nutrient feed bottle and pump.
Precision silicone peroxide tubing, with an ID of 4.8 mm (Cole-Parmer, Vernon Hills, IL, USA) was connected to the nutrient bottle attachment as shown in Figure 2.1 C. This tubing was divided into three using 6 mm barbed polypropylene cross connectors (Cole-Parmer) attached to smaller silicone tubing (precision peroxide silicone, ID 3.1 mm, Cole-Parmer). This tubing was reduced to Pharmed BPT tubing with an ID of 0.89 mm (Cole-Parmer) using a polyvinylidene fluoride (PVDF) barbed reducing connector (dimensions 7/32” and 13/16”, Cole-Parmer). The division and reduction of tubing is shown in Figure 2.1 D. The Pharmed tubing then passed through a Masterflex L/S 12-channel, 8-roller cartridge pump head attached to a Masterflex precision variable speed console drive (6–600 rpm, 230 V, Cole-Parmer) and was then adapted to silicone tubing (precision peroxide silicone, 3.1 mm ID, Cole-Parmer) using a reducing connector (PVDF, barbed, dimensions 7/32” and 13/16”, Cole-Parmer). This silicone tubing connected to brass tubes that entered the top of the biofilm apparatus and connected to the internal chambers. The inlet tube was positioned centrally, directly above the glass cup and 25 mm above the top of the glass beads. Waste flowed out through brass tubes, exiting the bottom of the internal chambers, which was connected to silicone tubing (precision peroxide silicone, ID 4.8 mm, Cole-Parmer) and allowed the waste to flow into a 5 L conical flask that acted as a waste reservoir. The overall set-up of the biofilm apparatus is shown in Figure 2.2.

2.3.2 Standard biofilm growth methods

Biofilms were fed 1/3 strength BHI with 1% sucrose. Antimicrobials to be tested were incorporated in the BHI and sucrose media. The biofilm apparatus was connected to nutrient media and flow started to prime the tubing with media. Priming was halted when media could be seen in the waste tubing. Glass cups with sintered glass bottoms, containing 1 g of glass beads, were sterilised by autoclaving in 100 ml glass beakers, five cups to a beaker. To each beaker 16 ml of BMM was added and cups were incubated at room temperature for 30 minutes. One hundred microlitres of overnight cultures of S. mutans 10449, S. oralis 34 and A. viscosus T14AV were added to each cup and beakers were incubated at 37°C for 30 minutes. Nine bead-containing glass cups were transferred to the biofilm apparatus, one to each of the nine internal chambers. The BMM was allowed to drain out and the nutrient flow was started at 0.8 ml/min/cup. The biofilm apparatus was incubated at 37°C in a temperature controlled room for the duration of the experiment (24, 48 or 72 hours). Following incubation, each glass cup was removed to a sterile glass beaker, 16 ml of cold PBS was added and pipetting was used to disrupt the biofilm.
Figure 2.2: Overall set-up of biofilm apparatus; showing nutrient bottles, tubing, pump and biofilm housing block.
Each beaker was sonicated in a 40 kHz ultrasonic cleaner (Unisonics, Labec Laboratory Equipment PTY LTD., Marrickville, NSW, Australia) for 30 seconds, held on ice for five minutes, then sonicated again for 30 seconds. The PBS was removed to a 50 ml plastic vial and 16 ml fresh PBS added to each beaker. Beakers were held on ice for five minutes, sonicated for 30 seconds and PBS transferred to the plastic vial. Tubes were centrifuged at 3200 x g for 20 minutes. Pellets were resuspended in 5 ml PBS and serially diluted 10-fold in PBS to 10^{-7}. Ten microlitres of each dilution was spotted three times onto CAB + rif, CAB + strep and CFAT agar. One hundred microlitres of undiluted cells were spread plated onto the three agar types. All plates were incubated at 37°C in air supplemented with 5% CO₂. CAB agar plates with antibiotics were incubated for 48 hours and CFAT agar plates for 60 hours. The total number of each bacterial species in the biofilms was enumerated.

### 2.3.3 Statistical analysis of biofilm results

All statistical analysis of results was carried out using GraphPad Prism 5 for Windows, Version 5.01 (GraphPad Software Inc. La Jolla, CA, USA). Comparison of two groups was done by unpaired t-test and comparison of multiple groups was done by one way analysis of variance (ANOVA) with a Tukey’s post test. Unless otherwise stated, values described as being significantly different differ at the 95% confidence level. Error on all graphs is the standard error of the mean.

### 2.3.4 Confirmation of strain designation by 16S PCR

A colony PCR to amplify the 16S gene of *S. mutans* 10449, *S. oralis* 34 and *A. viscosus* T14AV was carried out. Primers are listed in Table 2.2. Taq polymerase and 10 x Taq buffer were obtained from Roche. A reaction mix consisted of 34.5 μl MQ water; 5 μl 10 x Taq buffer; 5 μl 2 mM dNTPs; 1.5 μl forward primer and 1.5 μl reverse primer (final concentrations of 0.3 μM); and 1 μl Taq polymerase. Template DNA was added by plucking a colony with a sterile toothpick and dipping it in the reaction mix prior to the addition of the Taq polymerase. Toothpicks were then struck on CAB and incubated at 37°C for 48 hours. Amplification was performed with a thermocycler (Mastercycler, Eppendorf) using the following parameters: Cycle 1, denaturation temperature of 95°C for five minutes, annealing temperature of 33°C for 2.5 minutes, and extension temperature of 65°C for three minutes; cycles 2–31, denaturation temperature of 92°C for 30 seconds; annealing temperature of 33°C for 30 seconds, and extension temperature of 65°C for one minute. PCR products were
visualised on a 1% agarose gel. Products that were 800 bp in length were purified using a PCR purification kit (Axygen) according to the manufacturer’s instructions. The amount of product was determined using a Nanodrop spectrophotometer (Nanodrop Technologies). Products were then sequenced by the Massey DNA Sequencing Facility (Allen Wilson Centre) and sequencing reactions were prepared according to their instructions. The resulting sequence was analysed using SeqED software (DNASTAR Incorporated). Sequences were compared to published 16S sequences and the strain designation confirmed.

2.3.5 The use of selective agars

Overnight cultures of *S. mutans* 10448, *S. oralis* 34 and *A. viscosus* T14AV were serially diluted ten-fold to $10^{-7}$ in PBS. Ten microlitres of dilutions $10^{-4}$ to $10^{-7}$ were spotted three times onto CAB, CAB + rif, CAB + strep and CFAT agars. All plates were incubated at 37°C in air supplemented with 5% CO$_2$. CAB and CAB agar plates with antibiotics were incubated for 48 hours and CFAT agar plates for 60 hours. The total number of bacteria in each overnight culture was enumerated on both CAB (nonselective) and appropriate selective agars. This experiment was carried out in triplicate.

2.3.6 Comparison of spot- and spread-plating methods

Overnight cultures of *S. mutans* 10448, *S. oralis* 34 and *A. viscosus* T14AV were serially diluted ten-fold to $10^{-8}$ in PBS. Ten microlitres of dilutions $10^{-3}$ to $10^{-8}$ were spotted three times onto CAB agar. One hundred microlitres of dilutions $10^{-5}$ to $10^{-8}$ were spread onto CAB agar. The dilutions and spot and spread plating of each overnight culture was done in triplicate. Agar plates were incubated at 37°C in air supplemented with 5% CO$_2$ for 48 hours. The total number of bacteria in the overnight culture was enumerated using both the spread and spot plates.

2.3.7 Biofilms with 1/5 and 1/3 strength THB and BHI

The biofilm apparatus was set up as described in standard biofilm growth methods, with six cups. However, three cups were fed by 1/5 strength BHI supplemented with 1% sucrose and the remaining three by 1/5 strength THB supplemented with 1% sucrose. Following incubation cups were processed, diluted and plated as described in standard biofilm growth methods. The total number of each bacterial species in the biofilms was enumerated. This
experiment was repeated using 1/3 strength BHI supplemented with 1% sucrose and 1/3 strength THB supplemented with 1% sucrose as the nutrient source.

2.3.8 Bead conditioning methods

2.3.8.1 Amount of bacteria on inoculated beads
Three glass cups containing glass beads were prepared as described in standard biofilm growth methods. Excess BMM was drained from each cup and the cup was transferred to a sterile beaker. Sixteen millilitres of cold PBS was added and pipetting was used to disrupt the cells. Cells in each cup were harvested, diluted and plated as described in standard biofilm growth methods. The total number of each bacterial species in each cup was enumerated. This experiment was repeated for each of the bead conditioning methods, A to E, listed in Table 2.3.

2.3.8.2 Biofilms with varied bead conditioning methods
The biofilm apparatus was set up as described in standard biofilm growth methods with the exception that the beads were conditioned using methods A to E (Table 2.3). Following incubation for 48 hours, biofilm growth was recovered and enumerated as described in standard biofilm growth methods. Each bead conditioning method was done in triplicate. The five bead conditioning methods were done over two experiments, each with a triplet of beads conditioned with the control method.

2.3.9 Biofilms with no inoculum
The biofilm apparatus was set up as described in standard biofilm growth methods, with three cups, except no bacterial inoculum was added to the glass beads. The biofilm apparatus was incubated for 24 hours. Following incubation, beads were treated and cells diluted as described in biofilm growth methods. Each dilution was also plated onto CAB agar. This experiment was repeated with an incubation time of 48 hours.

2.3.10 Assay and experimental error
The biofilm apparatus was set up as described in standard biofilm growth methods with four cups. The biofilms were incubated for 24 hours and cups were harvested by sonication as described in standard biofilm growth methods.
Table 2.3: Bead conditioning methods

<table>
<thead>
<tr>
<th>Method name</th>
<th>Bead conditioning method</th>
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<tbody>
<tr>
<td>A</td>
<td>Beads were covered in 16 ml BMM for 30 minutes at room temperature (RT), inoculated and incubated at 37°C for 30 minutes.</td>
</tr>
<tr>
<td>B</td>
<td>Beads were covered in 16 ml BMM for 30 minutes at RT, excess BMM was drained and beads were dried at 37°C for 18 hours. Dried beads were inoculated and incubated at 37°C for 30 minutes.</td>
</tr>
<tr>
<td>C</td>
<td>Beads were covered in 16 ml BMM for 30 minutes at RT, inoculated and incubated 37°C for 18 hours.</td>
</tr>
<tr>
<td>D</td>
<td>Beads were inoculated and incubated at 37°C for 30 minutes.</td>
</tr>
<tr>
<td>E</td>
<td>Beads were covered in 16 ml BHI for 30 minutes at RT, inoculated and incubated at 37°C for 30 minutes.</td>
</tr>
</tbody>
</table>
Three cups were diluted and plated as described in standard biofilm growth methods. The fourth cup was also diluted and plated as described in standard biofilm growth methods; however, this dilution and plating was done three separate times. All plates were incubated at 37°C in air supplemented with 5% CO₂. CAB agar plates with antibiotics were incubated for 48 hours and CFAT agar plates for 60 hours. The total number of each bacterial species in each cup was enumerated.

**2.3.11 Viability of cells after sonication**

**2.3.11.1 Viability of bacterial cells**

Six sintered glass cups containing 1 g of glass beads were prepared in two beakers. To each beaker, 16 ml of BMM was added and cups were incubated at room temperature for 30 minutes. One hundred microlitres of overnight cultures of *S. mutans* 10449, *S. oralis* 34 and *A. viscosus* T14AV were added to each cup and beakers were incubated at 37°C for 30 minutes. Each cup was then placed in a separate sterile beaker and 16 ml PBS added. Three of the cups were sonicated in their beakers for 30 seconds, held on ice for five minutes, then sonicated for a further 30 seconds. The PBS was removed to a 50 ml plastic vial and 16 ml fresh PBS added to each beaker. The three beakers were then held on ice for a further five minutes, sonicated for 30 seconds and PBS transferred to the appropriate plastic vial. The remaining three beakers were held on ice during the initial sonication process (sonication-free control). The PBS was removed to a 50 ml plastic vial and 16 ml fresh PBS added to each beaker. The cups remained on ice while the first three cups were sonicated again and the PBS of the no sonication control cups was transferred to the appropriate plastic vial. All tubes were centrifuged, diluted and plated as described in standard biofilm growth methods.

**2.3.11.2 Viability of biofilm cells**

The biofilm apparatus was set up as described in standard biofilm growth methods, with three cups. The biofilms were incubated for 24 hours. Following incubation, each glass cup was removed to a sterile glass beaker, 16 ml of cold PBS was added and pipetting was used to dislodge the biofilm as much as possible without use of sonication. From each of the three beakers two 8 ml aliquots of the resuspended cells were transferred to fresh sterile beakers containing a sintered glass cup and 1 g of glass beads. To each cup-containing beaker a further 8 ml of cold PBS was added, to give a total of 16 ml in each beaker. This gave three pairs of beakers, one beaker of each pair to be sonicated and one as a sonication-free control. For the three beakers to be sonicated: Each beaker was sonicated for 30 seconds, held on ice
for five minutes, then sonicated for 30 seconds. The PBS was removed to a 50 ml plastic vial and 16 ml fresh PBS added to the three sonicated beakers. The sonicated beakers were then held on ice for five minutes, sonicated for 30 seconds and the PBS transferred to the appropriate plastic vial. For the sonication-free control beakers: The beakers were held on ice during the sonication of their paired sonicated beakers. The PBS was removed to a 50 ml plastic vial and 16 ml fresh PBS added to each beaker. The cups remained on ice while their paired beakers were sonicated for the second time. The PBS of the sonication-free control cups was transferred to the appropriate plastic vial. All tubes were centrifuged, diluted and plated as described in standard biofilm growth methods.

2.3.12 Removal of bacteria from glass beads by sonication

The biofilm apparatus was set up as described in standard biofilm growth methods, with three cups. The biofilms were incubated for 24 hours. Following incubation, each glass cup was removed to a sterile glass beaker, 16 ml of cold PBS was added and pipetting was used to dislodge the biofilm. Cups were then sonicated as described in standard biofilm growth methods. After the third sonication step and removal of the cells to a 50 ml plastic vial (sonications 1–3), a further 16 ml of PBS was added to the cups and they were held on ice for five minutes. Cups were sonicated for 30 seconds and the PBS removed to a plastic vial (sonication four). Sixteen millilitres of PBS was added to the beakers and they were held on ice for five minutes. Cups were sonicated for 30 seconds and the PBS removed to a 50 ml plastic vial (sonication five). All tubes were centrifuged, diluted and plated as described in standard biofilm growth methods.

2.3.13 Examination of sonicated beads by electron microscopy

The biofilm apparatus was set up as described in standard biofilm growth methods, with two cups. The biofilms were incubated for 48 hours. Following incubation the cups were processed as described in standard biofilm growth. A few unused beads, beads from which the biofilm had been removed by sonication, and beads with a control biofilm were transferred to separate glass tubes. Samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for one hour. Phosphate buffer (0.1 M) consisted of 11.36 g Na₂HPO₄ and 2.76 g NaH₂PO₄ dissolved in RO. The pH was adjusted to 7.4 and the volume made up to 1 L. Samples were then washed three times in 0.1 M phosphate buffer for 10 minutes per wash, and post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for one
hour and washed three times in 0.1 M phosphate buffer for 10 minutes per wash. Samples were then passed through an ethanol gradient of 25, 50, 75, 85 and 95%. They were placed in each ethanol concentration for 10 minutes and then washed three times in 100% ethanol for 10 minutes per wash. Samples were critical point dried in a Bal-Tec CPD-030 critical point dryer (Bal-Tec AG, Leica Microsystems GmbH, Wetzlar, Germany), then mounted on aluminium stubs with double-sided carbon tape and sputter coated with 5 nm gold palladium using an Emitech K575X Peltier-cooled high resolution sputter coater (EM Technologies Ltd, Kent, England). Samples were viewed in a JEOL JSM-6700F field emission scanning electron microscope (JEOL Ltd, Tokyo, Japan) at an accelerating voltage of 1.5 kV. A minimum of 10 fields were viewed for each biofilm condition examined.

2.3.14 Biofilm growth over time

The biofilm apparatus was set up as described in standard biofilm growth methods, with nine cups. The biofilm apparatus was incubated for 48 hours. Following 24, 36 and 48 hours incubation, three cups were removed from the biofilm apparatus and processed as described in standard biofilm growth methods.

This experiment was repeated and the biofilm incubated for 72 hours, with three cups removed from the biofilm and processed at 48, 60 and 72 hours.

2.4 Addition of antimicrobials to biofilms

2.4.1 Minimum inhibitory concentrations of lauricidin and zoocin A against planktonic cells

The MIC of lauricidin and zoocin A against S. mutans 10449, S. oralis 34 and A. viscosus T14AV was determined using 96-well flat bottom clear polystyrene microtitre plates (BD Falcon) incubated in an Infinite M200 plate reader (Tecan). Each well had a final volume of 200 μl comprising of 100 μl of bacterial inoculum (an overnight culture of the bacterial species to be tested, diluted 1:100 in BHI) and 100 μl of antimicrobial diluted in BHI. All concentrations described are the final concentration in the well after the addition of the bacterial inoculum and all experiments were done in triplicate. Lauricidin was serially diluted two-fold from 200 to 0.2 μg/ml. Zoocin A was serially diluted two-fold from 100 to 0.1 μg/ml for S. mutans and 500 to 0.68 μg/ml for S. oralis and A. viscosus. Each species
was also grown in BHI without any antimicrobial and with a matched ethanol and elution buffer control, with concentrations of ethanol and elution buffer which matched those of the highest concentration present in wells containing lauricidin and zoocin A respectively. Wells containing 200 μl of BHI alone and BHI with each antimicrobial at the highest concentration tested were also incubated without cell inoculum as negative controls. The three bacterial species were each tested against lauricidin and zoocin A individually. Once prepared, plates were incubated for 24 hours in an Infinite M200 plate reader (Tecan) at 37°C. An initial shaking step of five seconds (orbital shaking, 25 rpm/sec), followed by a five second wait was then followed by the OD₅₉₅ of each sample being recorded. The shaking and recording of the OD was repeated every hour during incubation.

2.4.2 Exposure of a biofilm to zoocin A after 12 hours of growth

The biofilm apparatus was set up as described in standard biofilm growth methods, with six cups. The biofilm apparatus was incubated for 24 hours. For the final 12 hours of incubation zoocin A was added to the nutrient feed of three cups at a final concentration of 40 μg/ml. Following incubation the cups were processed as described in standard biofilm growth methods.

2.4.3 Continuous exposure of a biofilm to zoocin A or lauricidin during incubation

The biofilm apparatus was set up as described in standard biofilm growth methods, with six cups. Antimicrobials were added to the nutrient feed of three cups for the entire incubation period of 24 hours. Zoocin A was added at final concentrations of 40, 80 and 120 μg/ml. Lauricidin was added at final concentrations of 10, 15 and 30 μg/ml. Following incubation the cups were processed as described in standard biofilm growth methods.

2.4.4 Addition of both lauricidin and zoocin A in combination

The biofilm apparatus was adjusted to have four 2 L bottles containing feed nutrients, each feeding two internal chambers housing cups. Bead-containing cups were prepared as described in standard biofilm growth methods and transferred to the biofilm apparatus. To the nutrient feed of each pair of cups was added zoocin A (final concentration 40 μg/ml); lauricidin (final concentration 10 μg/ml); both zoocin A (40 μg/ml) and lauricidin (10 μg/ml); or no addition was made. The biofilm apparatus was incubated for 24 hours. Following
incubation the biofilms were processed, as described in standard biofilm growth methods. This experiment was done a total of three times.

2.4.5 Biofilm growth over time, with and without antimicrobials

The biofilm apparatus was set up as described in standard biofilm growth methods, with nine cups. The biofilm apparatus was incubated for 72 hours. Following 24, 48 and 72 hours incubation, three cups were removed from the biofilm apparatus and processed as described in standard biofilm growth methods. A photograph was taken of the biofilms after 72 hours of incubation.

This experiment was repeated three more times with the addition of 40 μg/ml zoocin A, 10 μg/ml lauricidin and 40 μg/ml zoocin A with 10 μg/ml lauricidin to the feed medium.

2.4.6 Measuring the pH of biofilms

The biofilm apparatus was set up as described in standard biofilm growth methods, with nine cups. Zoocin A (40 μg/ml) was added to the nutrient feed of three cups and lauricidin (10 μg/ml) to the nutrient feed of a second three cups. The biofilm was incubated for 24 hours. Following incubation each glass cup was removed to a sterile glass beaker, 16 ml of cold MQ water was added and pipetting was used to disrupt the biofilm. Each beaker was sonicated for 30 seconds, held on ice for five minutes, and then sonicated again for 30 seconds. The MQ water was removed to a 50 ml plastic vial and 16 ml fresh MQ water added to each beaker. Beakers were held on ice for five minutes, sonicated for 30 seconds and the MQ water transferred to the plastic vial. Half of the resuspended cells were transferred to a separate 50 ml plastic vial, allowed to warm to room temperature and the pH was measured using a MP220 pH meter (Mettler Toledo, Greifensee, Switzerland). The remaining cells were centrifuged at 3200 x g for 20 minutes. Cells were resuspended in 5 ml PBS, diluted and plated as described in standard biofilm growth methods.

This experiment was repeated with zoocin A (40 μg/ml) and lauricidin (10 μg/ml) added to the nutrient feed medium in combination. It was also repeated with the zoocin A (40 μg/ml), lauricidin (10 μg/ml), and both in combination, with the biofilm incubation times increased to both 48 and 72 hours.
2.4.7 Use of fluorescent lectins to label *S. mutans* cells

Three 10 ml broths of each of BHI, BHI plus 1% sucrose, and BHI plus 1% glucose were inoculated with 500 μl of overnight culture of *S. mutans* 10449 and incubated at 37°C for 18 hours. The OD$_{595}$ of each culture was adjusted to 0.7. Five millilitres of each culture was centrifuged at 3200 x g for 15 minutes, the pellet was then resuspended in 1 ml PBS and transferred to a microcentrifuge tube. The cells in the microcentrifuge tube were centrifuged at 13,000 g for five minutes, the supernatant discarded and the pellet resuspended in 1 ml PBS. This wash step was repeated once more. Cells were resuspended in 1 ml PBS and 1 μl of 1 mg/ml FITC-WGA from *Triticum vulgaris* (Sigma) was added to give a final concentration of 1 μg/ml. Cells were incubated for one hour at room temperature in the dark. Following incubation, cells were centrifuged for five minutes at 13,000 x g, the supernatant was discarded and the cells resuspended in 1 ml PBS. This wash step was repeated twice more and then 200 μl of the resuspended cells were transferred in triplicate to a black 96-well flat bottom polystyrene microtitre plate (Nunc, Thermo Scientific). Three 200 μl volumes of PBS were used as a control. The fluorescence (in AU) of the cells was measured using an Infinite M200 plate reader (Tecan), with an excitation wavelength of 490 nm and emission wavelength of 525 nm. A 100 μl sample of each culture was serially diluted 10-fold in PBS to 10$^{-9}$. Ten microlitres of dilutions 10$^{-6}$ to 10$^{-9}$ was spotted three times onto CAB agar. Agar plates were incubated at 37°C in air supplemented with 5% CO$_2$ for 48 hours. The total number of bacteria in each culture was enumerated and the fluorescence per cell was calculated.

2.4.8 Labelling biofilm cells exposed to antimicrobials with fluorescent lectins

The biofilm apparatus was set up as described in standard biofilm growth methods, with nine cups. The biofilm was incubated for 72 hours. Following 24, 48 and 72 hours incubation, three cups were removed from the biofilm apparatus and placed in sterile beakers. To each beaker, 16 ml of cold MQ water was added and pipetting was used to dislodge the biofilm. Each beaker was sonicated as described in standard biofilm growth methods. Half of the resuspended cells were centrifuged at 3200 x g for 20 minutes. Cells were resuspended in 5 ml PBS. One millilitre of these cells were transferred to a microcentrifuge tube and washed, fluorescently labelled and the fluorescence measured, as described in section 2.4.7. A 100 μl sample of each culture was diluted and plated as described in standard biofilm growth methods. The total number of bacteria in each culture was enumerated and compared to
previous growth experiments. This experiment was repeated three more times with the addition of 40 μg/ml zoocin A, 10 μg/ml lauricidin, and 40 μg/ml zoocin A and 10 μg/ml lauricidin to the feed medium.

2.4.9 Examination of antimicrobial treated biofilms by electron microscopy

The biofilm apparatus was set up, inoculated and incubated as described in section 2.4.4. The biofilm apparatus was incubated for 48 hours. Following incubation a few beads from the each of the biofilm conditions were prepared and examined with a scanning electron microscope as described in section 2.3.13.

2.4.10 Addition of other antimicrobials to the biofilm model

The biofilm apparatus was set up as described in standard biofilm growth methods, with nine cups. Chlorhexidine digluconate (20% solution in water) was added to the nutrient feed of two triplets to give final chlorhexidine concentrations of 10 and 50 μg/ml. The biofilms were incubated for 24 hours. Following incubation, cells were resuspended and sonicated in MQ water as described in section 2.4.6. Half the resuspended cells were used to measure the pH of the biofilms while the remaining cells were centrifuged at 3200 x g for 20 minutes. The pellet was resuspended in 5 ml PBS and a 1 ml aliquot was transferred to a microcentrifuge tube, stained with FITC-WGA and the fluorescence measured as described in section 2.4.7. The remaining cells were diluted and plated as described in standard biofilm growth methods.

This experiment was repeated with the addition of 10 and 50 μg/ml of chlorhexidine with incubation times of 48 and 72 hours. It was also repeated with the addition of 1% Listerine FreshBurst antiseptic mouthwash and 1% Savacol antiseptic mouthwash to the nutrient feed with an incubation time of 24 hours.

2.4.11 Testing for the development of antimicrobial resistance in biofilm cells following antimicrobial exposure

The biofilm was set up as described above in section 2.4.4, with four 2 L bottles containing feed nutrients, each feeding two internal chambers housing cups. Bead containing cups were prepared as described in standard biofilm growth methods and transferred to the biofilm apparatus. To the nutrient feed of each pair of cups was added zoocin A (final concentration
40 μg/ml); lauricidin (final concentration 10 μg/ml); both zoocin A (40 μg/ml) and lauricidin (10 μg/ml); or no addition was made. The biofilm apparatus was incubated for 72 hours. Following incubation the biofilms were processed as described in standard biofilm growth methods.

CAB plates with lauricidin were prepared by supplementing agar with lauricidin after autoclaving to give a final concentration of 30, 120, 60, 90 or 180 μg/ml lauricidin. CAB plates with zoocin A were prepared by spreading the surface of CAB agar plates with 400 µl of 500, 1000, 2500, 5000, and 10,000 μg/ml zoocin A and allowing the liquid to absorb. This resulted in agar plates containing approximately 10, 20, 50, 100 and 200 μg/ml zoocin A.

Fifty colonies of each species from each biofilm condition (control, zoocin A, lauricidin and lauricidin and zoocin A in combination) were stabbed into CAB with 0, 30, 60, 90, 120 and 180 μg/ml lauricidin. The plate containing no lauricidin was stabbed last as a growth control. Fifty more colonies of each species from each biofilm condition were stabbed into CAB with 0, 10, 20, 50, 100 and 200 μg/ml zoocin A. The plate containing no zoocin A was stabbed last as a growth control. Agar plates were incubated for 60 hours at 37°C in air supplemented with 5% CO₂ and any stabs showing colonial growth were recorded as positive.

2.4.12 Further investigation of a colony displaying increased lauricidin resistance

The S. mutans colony that displayed increased resistance to lauricidin (designated colony A) was grown overnight in BHI with 0, 5, 10, 15, 30, 60, 120 and 180 μg/ml lauricidin at 37°C in air supplemented with 5% CO₂. The cultures with no lauricidin and the highest lauricidin concentration displaying growth were used in the methods described in section 2.4.1 to determine the MIC of the colony against lauricidin and compared to a culture of the original S. mutans strain. Concentrations of lauricidin tested ranged from 125 to 0.49 μg/ml, diluted from 125 μg/ml by serial two-fold dilution. This experiment was repeated to determine the MIC of these cultures against zoocin A.

Colony A was subcultured from the original 180 μg/ml lauricidin plate in section 2.4.11. It was stabbed into and struck onto 180 μg/ml lauricidin agar and CAB agar and used to inoculate a 10 ml BHI broth. All subcultures were incubated at 37°C in air supplemented with 5% CO₂. The agar plates were incubated for 72 hours and the BHI broth was incubated
for 24 hours. All subcultures that produced growth were then stabbed into 180 μg/ml lauricidin and incubated for 72 hours at 37°C in air supplemented with 5% CO₂.
3. Results: Production of zoocin A

3.1 Introduction

Zoocin A is produced by *S. equi* subspecies *zoopidemicus* 4881\(^{390}\) and can also be produced using a recombinant *E. coli* strain in shake flasks.\(^{247}\) However, production needs to be upscaled to produce the protein in sufficient quantities to use zoocin A as an antimicrobial in a biofilm model. Methods to quantify zoocin A are required to evaluate zoocin A production and ensure experimental consistency. It is important to quantify both the amount and activity of zoocin A, as it is possible for zoocin A to be present but not active. Measuring the activity of zoocin A also provides a method for quantifying zoocin A in solutions that have not been purified. Spectrophotometric assays are frequently used for protein quantification,\(^{325}\) while dye release\(^{501}\) or inhibition assays\(^{390}\) can be used to measure protein activity. The aim of this chapter was to examine methods to quantify both the activity and concentration of zoocin A samples and to upscale and optimise zoocin A production for use in a biofilm model.

3.2 Quantification of zoocin A

3.2.1 The Bradford assay

The Bradford assay was used as a protein assay for the quantification of zoocin A. It was performed three times on the same zoocin A sample with a BSA standard. In each case the \(r^2\) value of the standard curve created was above 0.99. However, the zoocin A concentrations determined ranged from 2555 to 43,010 µg/ml (Table 3.1), giving a standard deviation of 22,354 µg/ml. This large variation observed with the Bradford assay made it unsuitable for the quantification of zoocin A protein.

3.2.2 The Itzhaki assay

The Itzhaki assay was investigated as an alternative to the Bradford assay for zoocin A quantification. The Itzhaki assay was performed three times on the same zoocin A sample that was used for the Bradford assays, using BSA as a standard.
Table 3.1: Zoocin A concentrations determined by the Bradford and Itzhaki assays

<table>
<thead>
<tr>
<th>Protein assay</th>
<th>$r^2$ value</th>
<th>Zoocin A concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bradford Assay</td>
<td>0.9987</td>
<td>6299</td>
</tr>
<tr>
<td></td>
<td>0.9909</td>
<td>43,010</td>
</tr>
<tr>
<td></td>
<td>0.9943</td>
<td>2555</td>
</tr>
<tr>
<td>Itzhaki Assay</td>
<td>0.9922</td>
<td>5167</td>
</tr>
<tr>
<td></td>
<td>0.9970</td>
<td>5016</td>
</tr>
<tr>
<td></td>
<td>0.9985</td>
<td>5183</td>
</tr>
</tbody>
</table>
The $r^2$ value of each standard curve created was always above 0.99 and the sample replicates yielded very similar results (Table 3.1), with a standard deviation of 92 µg/ml. This made the Itzhaki assay much more reliable for the quantification of zoocin A than the Bradford assay. The Itzhaki assay was therefore used for quantification of zoocin A throughout this study.

### 3.2.3 Production of a zoocin A standard for protein quantification

Using zoocin A to create the standard curve when quantifying unknown zoocin A samples would allow the most accurate estimation of protein concentration. Therefore a zoocin A standard was made for use with the Itzhaki assay. Zoocin A was produced by small scale zoocin A production and protein purity was analysed by SDS-PAGE gel (Figure 3.1). The SDS-PAGE gel was deliberately overloaded to allow visualisation of contaminants present in low amounts. No other proteins could be seen in elution fractions one and two and these fractions were used for the production of a zoocin A standard. The standard was produced by dialysis and lyophilisation of these fractions. The activity of the zoocin A was tested before and after lyophilisation to check for any loss of activity caused by this process. Prior to dialysis the zoocin A sample had a volume of 10 ml and a titre of 8192 AU/ml, giving a total of 81,920 AU of zoocin A. Following dialysis the volume and titre had not changed. After lyophilisation the zoocin A was weighed and resuspended at 5 mg/ml. The total volume was 8.6 ml and the titre 8192 AU/ml, giving a total of 70,451 AU zoocin A. Since the spot test is only accurate within a factor of two, this suggests that the activity of zoocin A was probably not lost during the dialysis and lyophilisation process.

Quantification of a 25 µg/ml sample of the zoocin A standard, using the Itzhaki assay and a BSA standard, determined the amount of zoocin A as 6 µg/ml, suggesting using a BSA standard would lead to an underestimation of the amount of zoocin A by approximately four-fold. Therefore a zoocin A standard was used when calculating the concentration of zoocin A with the Itzhaki assay.

### 3.2.4 The dye release assay

The DRA and spot test can be used to quantify the activity of zoocin A. Zoocin A activity was measured in AU as the activity of the protein is not necessarily the same as the protein concentration.
Figure 3.1: SDS-PAGE gel of zoocin A purification. Lane 1: molecular weight standard; lane 2: cell supernatant; lane 3: cell supernatant passed over the Ni-NTA column; lanes 4 and 5: wash fractions 1 and 2; lanes 6 to 9: elution fractions 1 to 6; lane 10: molecular weight standard.
The DRA was used for zoocin A samples with a lower titre that could easily be diluted into the range of the DRA standard curve. Zoocin A samples with a very high titre were tested for activity by the spot test.

3.2.4.1 **Creating a standard curve for the dye release assay**

To use the dye release assay to quantify zoocin A activity a standard curve was required. It was found dye release was not linear for zoocin A concentrations above 10 AU/ml (Figure 3.2). A standard curve from 2.5 to 100 AU/ml gave an $r^2$ value of 0.09262 for a linear regression. At low concentrations of zoocin A (2.5 - 10 AU/ml) dye release was found to be more linear with an $r^2$ value of 0.9863 (Figure 3.3). For all future experiments zoocin A standard curves were made from 2.5 to 10 AU/ml.

3.2.4.2 **Length of incubation**

The DRA was carried out with varied incubation times to determine the time which gave the optimum standard curve. The $r^2$ value of a linear regression decreased with increased incubation time (Figure 3.4). An incubation time of 10 minutes resulted in an $r^2$ value above 0.99 and was therefore used in future DRA.

3.2.4.3 **Concentration of dye labelled substrate**

The DRA was carried out with varied concentrations of DLS to determine the optimum concentration for the assay. DLS with an OD$_{595}$ of 1.5 and 2.0 gave a linear regression with an $r^2$ value above 0.99 (Figure 3.5). DLS with an OD$_{595}$ of 2.0 gave the highest $r^2$ value and DLS used in future experiments had an OD$_{595}$ of 2.0.

3.2.4.4 **Standard curve for the dye release assay**

A standard curve was produced using the optimised methodology. A representative example is given as Figure 3.6. This standard curve had an $r^2$ value of 0.9048. The variation in dye release increased as the zoocin A concentration increased. This method was used in future DRA.
Figure 3.2: Dye release with the addition of zoocin A, using DLS with an OD\textsubscript{595} of 1.5 and an incubation time of 30 minutes. Linear regression gives an $r^2$ value of 0.09262.
Figure 3.3: Dye release over low zoocin A concentrations, using DLS with an OD$_{595}$ of 1.5 and an incubation time of 30 minutes. Linear regression gives an $r^2$ value of 0.9863.
Figure 3.4: DRA standard curve using DLS with an OD_{595} of 1.5. Dye release with varied incubation times where ◆ is 10 minutes ($r^2 = 0.9983$), ■ is 20 minutes ($r^2 = 0.9654$) and ▲ is 30 minutes ($r^2 = 0.9636$).
Figure 3.5: DRA standard curve with an incubation time of 10 minutes. Dye release with varied DLS concentrations where • is DLS with an OD of 1 ($r^2 = 0.9699$), ■ is DLS with an OD of 1.5 ($r^2 = 0.9896$), ♦ is DLS with an OD of 2 ($r^2 = 0.9983$) and ▲ is DLS with an OD of 3 ($r^2 = 0.9504$)
Figure 3.6: DRA standard curve with an incubation time of 10 minutes, using DLS with an OD595 of 2.0. Linear regression gives an $r^2$ value of 0.9048.
3.3 Production of zoocin A

3.3.1 Zoocin A production by S. equi subspecies zooepidemicus 4881

*S. equi* subspecies *zooepidemicus* 4881 is the original strain found to produce zoocin A. The amount of zoocin A that could be harvested from this strain was determined. The growth of *S. equi* subspecies *zooepidemicus* 4881 is seen in Figure 3.7. It took approximately 5.5 hours to reach an OD$_{595}$ of 0.7. Over the entire incubation time the *S. equi* subspecies *zooepidemicus* 4881 culture produced 3 AU/ml zoocin A, giving a total of 3000 AU per litre of culture.

3.3.2 Zoocin A production by E. coli M15 pQE-80L ZooA1, pREP4

*E. coli* M15 pQE-80L ZooA1, pREP4 is a recombinant producer of zoocin A. The amount of zoocin A that could be harvested from this strain, grown in two 500 ml shake flasks, was determined. The growth of *E. coli* M15 pQE-80L ZooA1, pREP4 is seen in Figure 3.7. *E. coli* M15 pQE-80L ZooA1, pREP4 grew faster than *S. equi* subspecies *zooepidemicus* 4881 and reached an OD$_{595}$ of 0.7 in 2.5 hours. The recombinant *E. coli* strain will only produce zoocin A after it is induced with IPTG, which occurred at an OD$_{595}$ of 0.7. The recombinant zoocin A producer strain produced 50 ml of 2048 AU/ml zoocin A after cell lysis and centrifugation, giving a total of 102,400 AU per litre of culture. Zoocin A purification resulted in 12 ml of 8192 AU/ml zoocin A, or a total of 98,304 AU per litre of culture. This zoocin A was from the first two elution fractions of the purification process. The reduction in the zoocin A titre from before to after zoocin A purification was accounted for by the presence of small amounts of zoocin A in other elution fractions. The purified zoocin A was quantified with the Itzhaki assay and contained 16 mg/ml, or a total of 192 mg of protein from one litre of culture.

3.3.3 Zoocin A production by E. coli M15 pQE-80L ZooA1, pREP4 over the induction period

Methods for the production of protein in a recombinant *E. coli* strain recommend five hours of incubation following the induction of the culture. To determine the optimum length of the post-induction incubation the amount of zoocin A produced over time was investigated.
Figure 3.7: Growth of zoocin A producing strains. OD₅₉₅ over time where – is *E. coli* M15 pQE-80L ZooA1, pREP4 grown in 500 ml shake flasks, ▲ is *E. coli* M15 pQE-80L ZooA1, pREP4 grown in a benchtop fermentor and ■ is *S. equi* subspecies *zooepidemicus* 4881 grown in 500 ml static flasks.
Most of the zoocin A was produced in the first hour of incubation following induction with IPTG (Figure 3.8). After three hours the amount of zoocin A produced per ml had nearly reached its maximum. Subsequent experiments for the production of zoocin A were incubated for four hours following induction with IPTG.

3.3.4 Production of zoocin A with varied IPTG concentrations

Recombinant expression systems frequently use the lac operator system de-repressed by the addition of IPTG to the culture. However, it has been suggested that IPTG can have a negative effect on bacterial growth rate and yield, especially at high concentrations. To minimise this possibility the minimum concentration of IPTG that could be used to induce the E. coli recombinant zoocin A producer strain was investigated. IPTG is usually used at a concentration of 1 mM, so a range of concentrations from 1 to 0.01 mM were tested. The growth rate of the culture was not altered by the varied IPTG concentrations (Figure 3.9). It was found that induction with 0.1 and 0.5 mM IPTG resulted in similar amounts of zoocin A as 1 mM IPTG, whereas induction of the culture with 0.01 mM IPTG resulted in reduced zoocin A production (Figure 3.10). Future experiments for the production of zoocin A were induced with 0.1 mM IPTG.

3.3.5 Production of zoocin A in a benchtop fermentor

Large amounts of zoocin A were required for use in the biofilm model. To produce zoocin A in sufficient quantities its production needed to be scaled up. The recombinant E. coli zoocin A producer strain was therefore grown in a 10 L volume in the BioFlo 410 benchtop fermentor. The culture took 3.5 hours to reach an OD595 of 0.7 (Figure 3.7), which was slightly slower than E. coli in a shake flask, although the fermentor culture reached a similar final OD. The culture produced 300 ml of 4096 AU/ml zoocin A, or 122,880 AU per litre of culture, after cell lysis and centrifugation. Zoocin A purification resulted in 100 ml of 8192 AU/ml zoocin A from the first two elution fractions, or 81,920 AU per litre of culture. This purified zoocin A was also quantified by Itzhaki assay and was calculated to have a concentration of 16,500 μg/ml, giving a total of 1.65 g zoocin A, or 165 mg per litre of culture.
Figure 3.8: Production of zoocin A by *E. coli* M15 pQE-80L ZooA1, pREP4 over the induction period.
Figure 3.9: Growth of *E. coli* M15 pQE-80L ZooA1, pREP4 with varied concentrations of IPTG. OD$_{595}$ over time where ■ is 1 mM IPTG, □ is 0.5 mM IPTG, ▲ is 0.1 mM IPTG and ◻ is 0.01 mM IPTG.
Figure 3.10: Production of zoocin A by *E. coli* M15 pQE-80L ZooA1, pREP4 with varied concentrations of IPTG. Amount of zoocin A produced over time where ▼ is 1 mM IPTG, ■ is 0.5 mM IPTG, ▲ is 0.1 mM IPTG and ▼ is 0.01 mM IPTG.
3.3.6 Optimisation of zoocin A production by E. coli M15 pQE-80L ZooA1, pREP4

3.3.6.1 Analysis of zoocin A genes for rare codons
The production of zoocin A by E. coli M15 pQE-80L ZooA1, pREP4 has never been optimised. It has been shown that codons in the recombinant gene sequence that occur with low frequency in E. coli can reduce the final protein yield. The codon frequency in the zoocin A sequence in the pQE-80L plasmid was therefore analysed and codons used with low frequency in E. coli identified. It was found that five codons were present in the zoocin A gene that are used in low frequency (in less than 10% of occurrences for the particular amino acid) in E. coli (Table 3.2). Of these, the AGA codon has been identified as most likely to reduce the efficiency of protein production, and an AGA codon occurs early in the zoocin A gene (second codon), which has been shown to increase the effect rare codons have on protein production. The first AGA codon was therefore changed to CGT (the most frequently used arginine codon in E. coli) by site-directed mutagenesis.

3.3.6.2 Site-directed mutagenesis of first AGA codon
The pQE-80L plasmid was amplified by PCR using primers designed to change the first AGA codon to CGT. Each PCR product consisted of a single band, corresponding in size to that of the plasmid with a zoocin A insert, and no bands were seen in the negative control reactions (Figure 3.11). Following Dpn I digestion, ligation and electroporation of the plasmid into E. coli DH5α, the resulting transformed colonies were tested for the presence of the zoocin A insert by colony PCR. The zoocin A gene was amplified and a 1000 bp band was seen from ten of the twenty colonies (Figure 3.12). The pQE-80L plasmid from four of these colonies was extracted and the zoocin A insert sequenced to confirm the codon change. Of these four colonies one colony had the codon change of AGA to CGT. This plasmid was designated pQE-80L ZooA1 CGT. The plasmid was electroporated into E. coli M15, pREP4 and the transformation of twelve colonies checked by colony PCR of the zoocin A insert. All colonies tested had the zoocin A plasmid insertion of the expected length of 1000 bp (Figure 3.13).

3.3.6.3 Screening for altered zoocin A production by strains altered by site-directed mutagenesis
The strains resulting from site-directed mutagenesis needed to be screened for altered zoocin A production.
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Codon</th>
<th>Frequency in E. coli *</th>
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Bold text indicates codons present in the zoocin A gene that occur with a frequency less than 0.1 in *E. coli*.

* Data from Wada et al., 1992.461
Figure 3.11: Agarose gel of a PCR of pQE-80L after amplification with mutagenic primers. Lane 1: 1 kb plus DNA marker; lanes 2 and 3: negative control; lanes 4 to 7: pQE-80L plasmid amplified with site directed mutagenesis primers. 4900 bp indicates the size of the predicted PCR products.
Figure 3.12: Agarose gel of a colony PCR of zoocin A insert in *E. coli* DH5α, pQE-80L. Lane 1: 1 kb plus marker; lanes 2 to 21: transformed cells 1 to 20; lane 22: positive control; lane 23: negative control. 1000 bp indicates the size of the predicted PCR products.
Figure 3.13: Agarose gel of a colony PCR of zoocin A insert in *E. coli* M15 pQE-80L ZooA1 CGT, pREP4. Lane 1: 1 kb plus marker; lanes 2 to 13: transformed cells 1 to 12; lane 14: 1 kb plus marker. 1000 bp indicates the size of the predicted PCR products.
Ten strains (transformants 1 to 10) were screened, as different protein production phenotypes can be seen between genetically identical strains after electroporation. The strains were screened for zoocin A activity so the protein did not have to be purified by Ni-NTA affinity column, as this results in the zoocin A being spread over several fractions. No increase in zoocin A production compared to the original strain was seen for any of the strains containing the pQE-80L ZooA1 CGT plasmid (Table 3.3) and no change in growth rate compared to the original producer strain was seen (Figure 3.14).

### 3.3.6.4 Insertion of pQE-80L ZooA1 and pREP4 into *E. coli* BL21-CodonPlus-(DE3)-RIL

Codon plus strains have a plasmid which contains genes for tRNA which correspond to codons that are rare in *E. coli* and are commonly found to reduce recombinant protein expression. Codon plus strains have been found to alleviate the effects of rare codons in some studies.\(^{24,73,224}\) *E. coli* BL21-CodonPlus-(DE3)-RIL (Stratagene) contains a pACYC based plasmid which contains extra copies of *E. coli* argU, ileY, and leuW tRNA genes which recognise the AGA/AGG, AUA, and CUA codons, respectively. The plasmids pQE-80L ZooA1 and pREP4 were electroporated into this strain. Six transformants (A to F) were isolated and checked for the presence of all three plasmids by digestion. For each control plasmid a single band was seen and for each transformant three bands were seen, each one corresponding with the band for a control plasmid (Figure 3.15).

### 3.3.6.5 Screening for altered zoocin A production by *E. coli* BL21-CodonPlus-(DE3)-RIL, pQE-80L ZooA1, pREP4 strains

The six *E. coli* BL21-CodonPlus-(DE3)-RIL, pQE-80L ZooA1, pREP4 transformants were screened for altered zoocin A production. No increase in zoocin A production compared to the original strain was seen for any of the transformants (Table 3.3). However, the transformants did have an increased growth rate and reached an optical density of 0.7 one hour faster than the original producer strain (Figure 3.16).

### 3.4 Conclusions

It was necessary to be able to quantify the amount of zoocin A in unknown samples. The Bradford assay was found to be unsuitable for the quantification of zoocin A. This assay utilises the binding of Coomassie brilliant blue dye to basic and aromatic amino acid residues to quantify protein and is known to only be linear at low protein concentrations.\(^{55}\)
Table 3.3: Production of zoocin A by *E. coli*

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<td></td>
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<td>Control strains</td>
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<tr>
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<td>Site directed mutagenesis strains</td>
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<td>M15 pQE-80L ZooA1 CGT, pREP4</td>
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</tr>
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<td>1</td>
<td>128</td>
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<td>Codon plus strains</td>
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</tr>
<tr>
<td>A</td>
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<tr>
<td>B</td>
<td>256</td>
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<tr>
<td>C</td>
<td>256</td>
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<tr>
<td>D</td>
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<tr>
<td>E</td>
<td>256</td>
</tr>
<tr>
<td>F</td>
<td>128</td>
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Figure 3.14: Growth of *E. coli* M15 pQE-80L ZooA1 CGT, pREP4 transformants screened for zoocin A production. OD$_{595}$ over time where ♦ is *E. coli* M15 pQE-80L ZooA1, pREP4; ← is *E. coli* M15 pREP4; and ♣, ♤, △, ▲, ●, ■, ▲, △, and ○ are *E. coli* M15 pQE-80L ZooA1 CGT, pREP4 transformants 1 to 10 respectively.
Figure 3.15: Agarose gel of a digestion of transformant plasmids with *Hind* III and *Bgl* II. Lane 1: 1 kb plus marker; lane 2: pQE-80L; lane 3: pREP4, lane 4: CodonPlus plasmid (DE3)-RIL; lanes 5 to 10: transformants A to F. 4900, 3400 and 3200 bp indicate the sizes of the predicted PCR products.
Figure 3.16: Growth of *E. coli* BL21-CodonPlus-(DE3)-RIL, pQE-80L ZooA1, pREP4 transformants screened for zoocin A production. OD$_{595}$ over time where * is *E. coli* M15 pQE-80L ZooA1, pREP4; is *E. coli* M15 pREP4; and * and * are *E. coli* BL21-CodonPlus-(DE3)-RIL, pQE-80L ZooA1, pREP4 transformants A to F respectively.
However, the Itzhaki assay, which measures the ultraviolet absorption of the complex formed between protein and copper in alkaline copper sulphate solutions, stains every amino acid and is always linear at any protein concentration.\(^{186}\) The Itzhaki assay was found to be more consistent for the quantification of zoocin A. Assays to measure zoocin A activity in a sample were also required, to ensure the protein used in future experiments was active. The spot test has been utilised previously; however, it is based on a doubling dilution and thus only large changes in zoocin A activity can be measured. It was therefore used to quantify the activity of high concentrations of zoocin A. The DRA was found to be linear over low zoocin A concentrations and the incubation times and DLS concentrations were optimised. The DRA was found to be suitable for the measurement of low zoocin A activity.

Zoocin A was first isolated from \textit{S. equi} subspecies \textit{zooepidemicus} 4881;\(^{390}\) however, production in this strain is very low and the protein is secreted so zoocin A accumulates in the culture supernatant in low concentrations. Production of zoocin A in a recombinant \textit{E. coli} strain concentrates the protein in the cell, and the cell lysate yielded over 30 times the amount of zoocin A per litre of culture than \textit{S. equi} subspecies \textit{zooepidemicus} 4881. This protein can easily be purified and concentrated by a Ni-NTA affinity column. It was found that production of zoocin A in a recombinant \textit{E. coli} strain can be shortened by reducing the post-induction incubation time to three hours, without loss of protein yield and that 0.1 mM IPTG was sufficient to induce the culture, allowing much less IPTG to be used, especially when inducing a 10 L culture. Production of zoocin A in 10 L volumes in a benchtop fermentor resulted in a similar yield per litre of culture as growth in 500 ml shake flasks, indicating protein yield was not reduced by the scaling up process. Several rare codons that had the potential to reduce protein expression in the recombinant strain were identified in the zoocin A gene. However, changing the first AGA codon in the gene to CGT or the insertion of pQE-80L ZooA1 and pREP4 plasmids into a codon plus strain containing genes for the rare codons AGA/AGG, AUA, and CUA did not result in a higher protein yield, but expression of zoocin A in the codon plus strain did reduce the production time.
4. Results: Development of a biofilm model

4.1 Introduction

Initial investigations into antimicrobial agents are often carried out on pure cultures of planktonic cells. However, oral bacteria form a biofilm, which leads to reduced bacterial susceptibility to antimicrobials and the effects of antimicrobials on biofilms can be limited to the upper layers of the biofilm. Therefore, to investigate the effects of antimicrobials on oral bacteria, a biofilm model must be used. Biofilm models range from single-species biofilms on glass slides or microtitre plates in nutrient broth to microcosms grown on sterilised enamel with artificial saliva and real-time monitoring of conditions. The biofilm model used therefore needs to balance realistic modelling of the in vivo environment with practicality and needs to be appropriate for the type of experiments being carried out. The aim of this chapter was to develop a simple triple species biofilm model, which can be used to investigate the effects of zoocin A and lauricidin on S. mutans.

4.2 Confirmation of biofilm model bacterial species by 16S PCR

To confirm the identity of bacterial species obtained from the culture collection (Department of Microbiology and Immunology, University of Otago), the 16S gene of S. mutans 10449, S. oralis 34 and A. viscosus T14AV was amplified by colony PCR. All three bacterial strains displayed a single product, 800 bp in size. The sequences of these PCR products were compared to published 16S sequences and in each case sequence data supported the bacterial species identification.

4.3 The use of selective agars

To enumerate individual bacterial species from the triple species biofilm, selective conditions must be used. It has been shown that selective conditions can cause a reduction in cell viability, so the growth of strains used in this study on selective agars was compared to their growth on nonselective agar. There was no significant difference between the cfu of S. mutans, S. oralis and A. viscosus on nonselective and the appropriate selective agar
No species grew on a selective agar designed to select for a different species. These selective conditions are therefore suitable for individual enumeration of the three species in the biofilm model.

### 4.4 Comparison of spot- and spread-plating methods

Spreading 100 μl of culture on an agar plate is commonly used to enumerate bacterial cells in a culture. An alternative is a spot plating method, in which 10 μl aliquots are dropped onto the agar surface, allowed to absorb and the cfu in each drop used to enumerate the bacterial culture. This method allows several dilutions to be plated on a single agar plate, reducing the agar required. The spot plate method was therefore compared to spread plating. No significant difference in the bacterial count of overnight cultures of *S. mutans*, *S. oralis* and *A. viscosus* was found when comparing spot- and spread-plating methods (Table 4.2). The spot plating method was used to enumerate bacteria in future experiments.

### 4.5 Biofilms with 1/5 and 1/3 strength THB and BHI

Different biofilm models use a range of different nutrient sources. To establish the conditions for the growth of the triple-species biofilm model, THB and BHI (two commonly used media) at 1/5 and 1/3 strength and all supplemented with 1% sucrose were compared. Both *S. mutans* and *S. oralis* had significantly higher growth in 1/3 strength BHI compared to 1/5 strength BHI (Figure 4.1). *A. viscosus* did not grow in 1/3 THB, but no significant differences in growth were seen in the levels of *A. viscosus* with the other nutrient broths. Biofilms grown in THB had greater variation between replicates than biofilms grown in BHI and no differences between 1/3 and 1/5 strength THB could be seen for *S. mutans* and *S. oralis*. Future biofilm experiments were carried out with 1/3 strength BHI supplemented with 1% sucrose.

### 4.6 Varied bead conditioning methods

Preparation of the biofilm surface varies greatly between biofilm models. Different bead conditioning methods were therefore compared.
Table 4.1: Strain viability on selective and nonselective agars

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<th>Bacterial species</th>
<th>Viable count (log cfu/ml [± SEM]) when grown on:</th>
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<td></td>
<td>CAB</td>
<td>CAB + rif</td>
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<tr>
<td>S. mutans 10449</td>
<td>8.9 (± 0.047)</td>
<td>8.9 (± 0.015)</td>
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<tr>
<td>S. oralis 34</td>
<td>9.0 (± 0.014)</td>
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<tr>
<td>A. viscosus TI4AV</td>
<td>8.6 (± 0.076)</td>
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### Table 4.2: Strain enumeration by spot- and spread-plating methods

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<td>Spot method</td>
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<td>S. mutans 10449</td>
<td>8.8 (± 0.072)</td>
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<tr>
<td>S. oralis 34</td>
<td>8.6 (± 0.074)</td>
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<tr>
<td>A. viscosus TI4AV</td>
<td>8.3 (± 0.050)</td>
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</table>
Figure 4.1: Biofilms grown with different nutrient broths. Bacterial count per bead-containing cup after 24 hours where is S. mutans 10449, is S. oralis 34 and is A. viscosus TI4AV.
4.6.1 Amount of bacteria on inoculated beads with varied bead conditioning methods

The numbers of bacteria attaching to glass beads conditioned with different methods was compared. All bead conditioning methods resulted in similar levels of bacteria on the beads (Figure 4.2). However, because the amount of bacteria on the inoculated beads was very consistent, small, yet significant, differences could be detected. Bead conditioning method D involved the inoculation of dry, unconditioned beads and resulted in significantly less bacteria of each species on the beads compared to methods B, C and E. Methods B, C and E all involved coating the beads, either in BMM (B and C) or BHI (E) and method B included drying the beads after coating, while methods C and E were not dried. Method C, which was incubated overnight following inoculation, had the highest levels of bacteria and had significantly more bacteria than methods A and D.

4.6.2 Biofilms with varied bead conditioning methods

As the varied bead conditioning methods resulted in very similar levels of bacteria attaching to the glass beads, the biofilm growth after 24 hours with different bead conditioning methods was compared. None of the bead conditioning methods resulted in significantly different levels of *S. mutans* 10449 or *A. viscosus* TI4AV after 24 hours of incubation (Figure 4.3). *S. oralis* 34 did not survive at detectable levels on unconditioned beads in method D. Bead conditioning methods B and D both involved dry beads and resulted in more variable bacterial counts between the replicates. Conditioning method E, using BHI instead of BMM, resulted in significantly less *S. oralis* than methods A and C. Bead conditioning method A was used in future experiments as the initial levels of bacteria on these beads was lower than for beads inoculated using method C (reported in section 4.5.1) and *S. oralis* and *S. mutans* had therefore increased in numbers further in 24 hours of incubation with method A compared to method C.

4.7 Biofilms with no inoculum

The biofilm apparatus was prepared and incubated with no bacterial inoculum to ensure it was not prone to contamination during incubation, as contamination may remain undetected due to the selective conditions used to enumerate the bacteria. The biofilms incubated for 48 hours with no inoculum produced no colonies on any of the agar plates.
Figure 4.2: Inoculated beads with varied conditioning methods. Bacterial count per bead-containing cup where □ is *S. mutans* 10449, ○ is *S. oralis* 34 and ▲ is *A. viscosus* TI4AV and bead conditioning methods A to E are listed below.

A: Beads were covered in 16 ml BMM for 30 minutes at RT, inoculated and incubated at 37°C for 30 minutes.

B: Beads were covered in 16 ml BMM for 30 minutes at RT, excess BMM was drained and beads were dried at 37°C for 18 hours. Dried beads were inoculated and incubated at 37°C for 30 minutes.

C: Beads were covered in 16 ml BMM for 30 minutes at RT, inoculated and incubated 37°C for 18 hours

D: Beads were inoculated and incubated at 37°C for 30 minutes.

E: Beads were covered in 16 ml BHI for 30 minutes at RT, inoculated and incubated at 37°C for 30 minutes
Figure 4.3: Biofilms with varied bead conditioning methods. Bacterial count after 24 hours where □ □ is *S. mutans* 10449, □ □ is *S. oralis* 34 and □ □ is *A. viscosus* TI4AV and bead conditioning methods A to E are listed below.

A: Beads were covered in 16 ml BMM for 30 minutes at RT, inoculated and incubated at 37°C for 30 minutes.

B: Beads were covered in 16 ml BMM for 30 minutes at RT, excess BMM was drained and beads were dried at 37°C for 18 hours. Dried beads were inoculated and incubated at 37°C for 30 minutes.

C: Beads were covered in 16 ml BMM for 30 minutes at RT, inoculated and incubated 37°C for 18 hours

D: Beads were inoculated and incubated at 37°C for 30 minutes.

E: Beads were covered in 16 ml BHI for 30 minutes at RT, inoculated and incubated at 37°C for 30 minutes
The biofilms incubated for 24 hours had a single white, matt, colony on two CAB agar plates and two yellow colonies with β-haemolysis on one CFAT agar plate. These colonies were all from the $10^1$ dilution. No other bacterial growth was observed on any of the agar plates. The waste media for both biofilm experiments with no inoculum was clear and did not display any bacterial growth. These results indicate the biofilm is not contaminated with high levels of bacteria and that when the beads were inoculated with \textit{S. mutans}, \textit{S. oralis} and \textit{A. viscosus} they were not also growing contaminating species.

### 4.8 Assay and experimental error

When a biofilm model is exposed to different antimicrobial conditions the results must be consistent enough to allow any differences in bacterial growth to be seen. Assay error is the error inherent in the experimental system, which results from unavoidable errors in the quantitative assay. This can be determined by carrying out the quantitation procedure on a single sample multiple times. Experimental error is the variation that occurs between experimental replicates and is determined by performing the entire experiment multiple times. Both the experimental and assay error were investigated to determine the degree of variability within biofilm replicates. In each case the variability was minimal (Figure 4.4), indicating changes in the biofilm growth would be detectable. The greatest variability was observed for experimental replicates as this also includes the assay error.

### 4.9 Viability of cells after sonication

It was necessary to compare the viability of \textit{S. mutans}, \textit{S. oralis} and \textit{A. viscosus} before and after sonication to check the biofilm cell viability was not being reduced by the cell recovery process. No difference between the bacterial count of cells with and without sonication was seen for the non-biofilm cells, or the \textit{S. oralis 34} and \textit{A. viscosus} T14AV biofilm cells (Figure 4.5). The bacterial count of \textit{S. mutans} biofilm cells was significantly decreased in the sonication-free control when compared to the sonicated cells.

### 4.10 Removal of bacteria from glass beads by sonication

A fourth and fifth sonication step was carried out to investigate the amount of bacterial cells that remained on the glass beads following the removal of the biofilm cells for enumeration.
Figure 4.4: Assay and experimental error. Bacterial count per bead-containing cup after 24 hours where ■ is three replicates each processed individually (experimental error) and ● is one cup processed three times (assay error).
Figure 4.5: Viability of cells after sonication. Bacterial count per bead-containing cup after sonication where  is cells that have been sonicated and  is cells that have not been sonicated. A is non-biofilm cells and B is biofilm cells.
The initial three sonication steps removed 2.5 logs more *S. mutans* than the fourth sonication step, and 3.5 logs more than a fifth sonication step (Figure 4.6). For both *S. oralis* and *A. viscosus* the reduction was even greater. No more *A. viscosus* was isolated from the beads in the fourth and fifth sonication steps. This result suggests that over 99% of the bacteria cells were removed from the beads by the initial three sonication steps.

### 4.11 Examination of cleaned beads by electron microscopy

To confirm that the majority of biofilm cells were removed from the glass beads by three sonication steps beads were examined by electron microscopy. Both the unused beads and those from which biofilm cells had been removed by sonication did not have any bacterial growth on them when examined by scanning electron microscopy (Figure 4.7), while the control biofilm beads had very visible bacterial growth covering a large proportion of the bead. This result established that three sonication steps were sufficient to remove the biofilm cells from the glass beads.

### 4.12 Biofilm growth over time

Biofilm growth over 72 hours was measured to investigate the stability of the bacteria in the biofilm model. Over the 72 hours the level of *S. mutans* increased steadily from the number of bacteria on the inoculated beads and plateaued around 48 hours at about 9.5 logs (Figure 4.8). *A. viscosus* levels decreased during the initial 24 hours and then appeared to stabilise at about 3.5 logs. Over the first 36 hours *S. oralis* 34 levels increased at levels similar to *S. mutans*. However, they then sharply declined over the following 36 hours and after 72 hours were at a similar level to *A. viscosus*. Future time course experiments were sampled at 24, 48 and 72 hours.

### 4.13 Conclusions

In this chapter preliminary experiments for developing a biofilm model were carried out. The final methods that are outlined in section 2.3.2 have been shown to be consistent over several replicates, with low assay and experimental error, which allowed relatively small differences in biofilm composition resulting from antimicrobial addition to be seen.
Figure 4.6: Removal of bacteria from glass beads by sonication. Bacterial count of bacteria isolated per sonication where □ is sonications 1-3, ■ is a 4th sonication and ● is a 5th sonication.
Figure 4.7: Scanning electron microscope images of a control biofilm, sonicated beads and unused glass beads. Column A is beads with a control biofilm after 48 hours of incubation, column B is beads with a control biofilm after 48 hours of incubation from which cells have been recovered by sonication and column C is unused beads. Rows 1, 2 and 3 are magnifications of 25, 85 and 1000 x respectively.
Figure 4.8: Biofilm growth over 72 hours. Bacterial count per bead-containing cup over time where – is *S. mutans* 10449, ■ is *S. oralis* 34 and – is *A. viscosus* TI4AV.
The bacterial cells were removed from the glass beads by three sonication steps and it was shown that this sonication does not reduce the viability of the cells and was sufficient to remove the bacterial cells from the beads. The three bacterial species were individually enumerated by the use of selective agars and a comparison of these agars with the nonselective CAB agar indicated there was no difference in the viability of *S. mutans*, *S. oralis* or *A. viscosus* grown on CAB and the appropriate selective agars. It was found that results gained by the spot plating method did not differ from the spread plating method, so the spotting method was used to conserve agar plates. BHI was found to give more consistent bacterial growth results compared to THB. With 1/3 strength BHI the levels of *S. mutans* and *S. oralis* were maintained compared to the inoculum (reported in section 4.5.1). However, with 1/5 strength BHI the bacterial count of all three species was decreased compared to the initial inoculum. BHI at 1/3 strength was therefore used for future experiments. No major differences were found in the amount of bacteria present on the beads when using varied bead conditioning methods. However, bead conditioning method A (involving coating the cells in BMM, inoculating the beads and allowing cells 30 minutes to attach) was found to produce consistent results, where both *S. mutans* and *S. oralis* increased in the first 24 hours of incubation, and was therefore used in future experiments. Beads that were incubated with no inoculum did not grow a biofilm, indicating the apparatus is not prone to contamination. Using the final method outlined in section 2.3.2, *S. mutans* was found to increase over time in the biofilm, while *A. viscosus* decreased slowly. *S. oralis* was found to increase initially, over the first 36 hours, and then started to decline. This biofilm model was then used to investigate the effects of antimicrobials on biofilm cells.
5. Results: Addition of antimicrobials to the biofilm model

5.1 Introduction

Zoocin A is an endopeptidase$^{138}$ that ruptures of the bacterial cell wall$^{391}$ and is active against \textit{S. mutans}$^{188,390}$. Lauricidin is a distilled monoglyceride that acts upon the cell membrane$^{46}$ and has antimicrobial activity against a range of gram-positive bacteria, including streptococci.$^{202,203}$ To investigate the effects of these antimicrobials against oral bacteria a biofilm model must be used. The development of a triple-species biofilm model was described in chapter four. The aim of this chapter was to use the biofilm model to investigate the effects of zoocin A and lauricidin, both individually and in combination, upon a biofilm and compare these results with the effects of the chlorhexidine and the commercially available anti-plaque agents Savacol and Listerine antiseptic mouthwashes.

5.2 The minimum inhibitory concentration of zoocin A and lauricidin in broth

To establish a starting point for the concentrations of zoocin A and lauricidin required to kill the three bacterial species’ used in the biofilm model, the MIC of each species in broth was determined. Both \textit{S. oralis} 34 and \textit{A. viscosus} T14AV were considered resistant to zoocin A as they were not inhibited by concentrations up to 500 μg/ml. The MIC of zoocin A for \textit{S. mutans} 10449 was 6.25 μg/ml. The MIC of lauricidin for \textit{S. mutans} 10449 was 25 μg/ml, while for \textit{S. oralis} 34 and \textit{A. viscosus} T14AV it was 12.5 μg/ml. No growth was observed in uninoculated BHI or BHI with zoocin A or lauricidin. Zoocin A was stored in elution buffer and lauricidin in 95% ethanol. No alteration in growth was seen for each species grown with an ethanol or elution buffer control at a concentration matched to that present for the highest antimicrobial concentration tested.

5.3 Exposure of a biofilms to zoocin A

5.3.1 Zoocin A for the final 12 hours of a 24 hour biofilm

Zoocin A was added to the biofilm growth medium for the final half of incubation to investigate whether zoocin A could inhibit \textit{S. mutans} in a biofilm that was already established.
No significant difference in the levels of *S. mutans* 10449, *S. oralis* 34 or *A. viscosus* T14AV was seen between control biofilms and those exposed to 40 μg/ml zoocin A for the final 12 hours of a 24 hour incubation period (Figure 5.1). For all further experiments antimicrobials were included for the entire incubation period.

### 5.3.2 Zoocin A for the entire incubation period

Different concentrations of zoocin A were added to the biofilm for the entire incubation period to establish what concentration of zoocin A was required to inhibit growth of *S. mutans* 10449 in the biofilm model. *S. mutans* 10449 was significantly decreased by all concentrations of zoocin A tested (Figure 5.2). The bacterial count of *S. mutans* 10449 after exposure to 120 μg/ml zoocin A was also significantly lower than after exposure to 40 μg/ml. There was no significant difference between the bacterial count of *S. mutans* 10449 after exposure of to 80 μg/ml and exposure to 120 or 40 μg/ml. The amount of *S. oralis* 34 increased with zoocin A exposure, and after exposure to 120 μg/ml was significantly higher than in the control biofilm. No change in the amount of *A. viscosus* T14AV in the biofilms was seen.

### 5.4 Exposure of biofilms to lauricidin

Lauricidin was added to the biofilm to establish what concentration of lauricidin was required to inhibit biofilm growth. No significant difference in the growth of *S. mutans* 10449, *S. oralis* 34 or *A. viscosus* T14AV was seen between the control and an ethanol control matched to the concentration of ethanol present in the highest concentration of lauricidin (Figure 5.3). The higher concentrations of lauricidin, 30 and 15 μg/ml, resulted in a significant reduction in all three species, and a much larger reduction in *S. mutans* 10449 than 40 μg/ml zoocin A. Biofilms exposed to 10 μg/ml lauricidin significantly reduced *S. mutans* 10449 by almost three logs, while levels of *S. oralis* 34 and *A. viscosus* were not significantly reduced. In future experiments lauricidin was added at a final concentration of 10 μg/ml, as this concentration reduced the levels of *S. mutans* 10449 in the biofilm without reducing *S. oralis* 34 and *A. viscosus* T14AV. Higher concentrations of lauricidin also reduced the bacterial levels to close to the detection limit of the experiment which increased the experimental variation.
Figure 5.1: Biofilm with 12 hours of zoocin A exposure. Bacterial count of the biofilms after 24 hours where is the control biofilm and is exposure to 40 µg/ml zoocin A for the final 12 hours of incubation.
Figure 5.2: Biofilms exposed to zoocin A. Bacterial count of the biofilms where ✧ is the control and ✦, ☉ and ☾ are exposure to 40, 80 and 120 µg/ml zoocin A respectively.
Figure 5.3: Biofilms exposed to lauricidin. Bacterial count of the biofilms after 24 hours where ■ is the control, □ is an ethanol control, and ■■■■■■ and ■■■■ are exposure to 10, 15 and 30 µg/ml lauricidin respectively.
5.5 Exposure of biofilms to zoocin A and lauricidin in combination

Biofilms were also treated with zoocin A and lauricidin in combination. *S. mutans* 10449 was significantly reduced by one to two logs with exposure to zoocin A (40 μg/ml) and lauricidin (10 μg/ml) individually (Figure 5.4). The levels of *S. mutans* 10449 in biofilms exposed to zoocin A or lauricidin were not significantly different from each other. When zoocin A and lauricidin were used in combination *S. mutans* 10449 was reduced further, by nearly four logs compared to the untreated control biofilms. This reduction in *S. mutans* 10449 was significant when compared to the untreated control biofilm and those exposed to zoocin A and lauricidin individually. The bacterial count of *S. oralis* 34 was unchanged compared to the control, when biofilms were exposed to either lauricidin, or zoocin A and lauricidin in combination. When biofilms were exposed to zoocin A *S. oralis* 34 was significantly higher than the other biofilm conditions. No change in the levels of *A. viscosus* T14AV was seen.

5.6 Exposure of biofilms to antimicrobials for up to 72 hours

To investigate the effects of the antimicrobials over a longer period of time biofilms were grown and harvested at 24, 48 and 72 hours. The highest levels of bacteria were seen with *S. mutans* 10449 in the control biofilm, where they increased over the 72 hour incubation period (Figure 5.5). While a small reduction in the levels of *S. mutans* 10449 was seen with the addition of zoocin A and lauricidin to the biofilms individually, a major reduction at all three time points was seen when zoocin A and lauricidin were added to the biofilm in combination. When zoocin A was added to the biofilms an increase in the amount of *S. oralis* 34 was seen, with the bacterial count being significantly higher than the control biofilm at 48 and 72 hours. This pattern was repeated when comparing the levels of *S. oralis* 34 in control biofilms and those exposed to zoocin A and lauricidin in combination. Overall, the biofilms exposed to antimicrobials had higher levels of *S. oralis* 34 than that of the control, although bacterial levels declined over time. After 72 hours of incubation the control biofilm had the lowest levels of *S. oralis* 34, which was significantly reduced compared to all other biofilm conditions. Biofilms treated with zoocin A had the highest levels of *S. oralis* 34, significantly higher than the other biofilm treatments. The difference in *S. oralis* 34 cfu for biofilms treated with lauricidin or both zoocin A and lauricidin in combination were also significantly different from the other biofilm treatments.
Figure 5.4: Biofilms exposed to zoocin A, lauricidin and both in combination. Bacterial count of the biofilms after 24 hours where ■ is the control, □□□ is exposure to 40 µg/ml zoocin A, □□□□ is exposure to 10 µg/ml lauricidin, and □□□□□ is exposure to zoocin A (40 µg/ml) and lauricidin (10 µg/ml) in combination.
Figure 5.5: Biofilms exposed to antimicrobials over 72 hours. Bacterial count of the biofilms where ✷ is the control, ☐ is exposure to 40 µg/ml zoocin A, ☐ is exposure to 10 µg/ml lauricidin, and ☐ is exposure to zoocin A (40 µg/ml) and lauricidin (10 µg/ml) in combination. A, B and C are the bacterial counts of *S. mutans*, *S. oralis* and *A. viscosus* respectively.
The bacterial count of *A. viscosus* T14AV also declined over time, with the control biofilm having significantly less *A. viscosus* T14AV than the biofilms exposed to antimicrobials after 48 and 72 hours. The addition of lauricidin to the biofilms initially resulted in the largest decrease in *A. viscosus* T14AV, with levels significantly reduced compared to biofilms exposed to zoocin A or zoocin A and lauricidin in combination. However, in biofilms exposed to lauricidin the levels of *A. viscosus* T14AV increased over time to reach those of the biofilms exposed to other antimicrobial combinations.

After 72 hours the appearance of the biofilms was recorded (Figure 5.6). The control biofilm was thick, covering all the beads and the inside of the glass cup. The zoocin A or lauricidin biofilms had less biofilm growth, although it still covered the glass beads. The cup exposed to zoocin A and lauricidin did not have visible growth, although following sonication the PBS was cloudy with resuspended cells and still had measurable levels of all three bacterial species.

**5.7 The pH of biofilms exposed to zoocin A and lauricidin**

The pH of the biofilm cells was measured, as it is known that the low pH in an oral biofilm after sucrose exposure contributes to the erosion of tooth enamel.268,354 The pH of the control biofilms decreased over time, from 5.4 after 24 hours to 4.3 after 72 hours (Figure 5.7). At all three time points, the pH of the control biofilm was significantly less than that of the biofilms exposed to antimicrobials. Biofilms exposed to both lauricidin and zoocin A in combination retained a pH of over 7.0 throughout the incubation period. Biofilms exposed to zoocin A or lauricidin individually had a pH above 7.0 after 24 hours which reduced to 6.2 and 6.6 respectively after 72 hours. After 72 hours no difference could be seen between zoocin A and lauricidin individually, but both had a significantly lower pH than biofilms exposed to zoocin A and lauricidin in combination.

**5.8 Use of fluorescent lectins to label *S. mutans* cells**

The fluorescent lectin FITC-WGA has been used to stain biofilms and measure the reduction in biofilm formation after antimicrobial exposure.67 To confirm FITC-WGA can be used to measure biofilm production the fluorescence of *S. mutans* 10449 cells grown with different sugars was tested.
Figure 5.6: Biofilms after 72 hours, with or without antimicrobial exposure. A is the control biofilm with no antimicrobial added, and B, C and D are biofilms exposed to zoocin A (40 µg/ml), lauricidin (10 µg/ml), and zoocin A (40 µg/ml) with lauricidin (10 µg/ml) respectively.
Figure 5.7: The pH of biofilm cells over 72 hours. pH of the biofilm cells where ■ is the control, ▲ is exposure to 40 µg/ml zoocin A, ▼ is exposure to 10 µg/ml lauricidin, and ◀️ is exposure to zoocin A (40 µg/ml) and lauricidin (10 µg/ml) in combination.
When grown in the presence of 1% sucrose *S. mutans* 10449 cells labelled with FITC-WGA produce significantly more fluorescence than cells grown with 1% glucose or in BHI without additional sugars (Figure 5.8). This result indicates *S. mutans* 10449 cells growing in a biofilm and labelled with FITC-WGA will produce more fluorescence than non-biofilm cells.

### 5.9 Labelling biofilm cells exposed to antimicrobials with fluorescent lectins

It was noted that visually the biofilms appeared greatly reduced when exposed to antimicrobials, even though cell counts suggested the reduction in numbers of some of the bacterial species was limited. To quantify this FITC-WGA was used to measure the biofilm formation of the cells exposed to zoocin A, lauricidin and both in combination. The control biofilm had a significantly higher level of fluorescence than the antimicrobial treated biofilms at all time points measured (Figure 5.9). No difference could be seen between the different antimicrobial treatments.

### 5.10 Examination of antimicrobial treated biofilms by electron microscopy

To confirm the changes in biofilm formation seen with the fluorescent lectin staining, biofilms were examined by scanning electron microscopy. Control biofilms were thick and coated most of the beads’ surface (Figure 5.10). A dense and continuous microbial mat could be seen, consisting almost entirely of cocci. Biofilms treated with zoocin A appeared to be broken into large pieces, with areas where the biofilm was cracked and open. Biofilms treated with lauricidin were thinner than the control biofilms, had holes throughout the biofilm and less of the beads’ surface was covered in microbial cells. Biofilms treated with lauricidin and zoocin A in combination were thin and very little microbial growth was visible on the beads.

### 5.11 Addition of chlorhexidine to the biofilm model

Chlorhexidine has been shown to be an effective antimicrobial for the oral cavity. The effect of chlorhexidine on the biofilm model was investigated to compare the effects of zoocin A and lauricidin to an established antimicrobial. After 24 hours the levels of *S. mutans* 10449 was significantly decreased by 10 and 50 μg/ml chlorhexidine, while *S. oralis* 34 and *A. viscosus* T14AV were only significantly reduced by 50 μg/ml chlorhexidine (Figure 5.11).
Figure 5.8: Fluorescence of FITC-WGA labelled *S. mutans* cells. Fluorescence per colony forming unit under varied growth conditions.
Figure 5.9: Fluorescence of FITC-WGA labelled biofilms cells exposed to antimicrobials. Fluorescence of the biofilms over time where ◦ is the control, ▲ is exposure to 40 µg/ml zoocin A, ▼ is exposure to 10 µg/ml lauricidin, and ◖ is exposure to zoocin A (40 µg/ml) and lauricidin (10 µg/ml) in combination.
Figure 5.10: Scanning electron microscope images of biofilms after 48 hours with and without antimicrobials. Row A is the control biofilm and rows B, C and D are biofilms exposed to zoocin A (40 μg/ml), lauricidin (10 μg/ml), and zoocin A (40 μg/ml) with lauricidin (10 μg/ml) respectively. Columns 1, 2 and 3 are magnifications of 85, 1000 and 3000 x respectively.
Figure 5.11: Biofilm growth with chlorhexidine over 72 hours. Bacterial count of the biofilms where • is the control, ■ is exposure to 10 µg/ml chlorhexidine and ▲ is exposure to 50 µg/ml chlorhexidine. A, B and C are the bacterial counts of S. mutans 10449, S. oralis 34 and A. viscosus T14AV respectively.
With the addition of 50 \( \mu g/ml \) chlorhexidine, levels of all three species were reduced to below the detection limit after 24 hours. After 48 hours, \textit{S. mutans} 10449 and \textit{A. viscosus} T14AV were significantly reduced by 50 \( \mu g/ml \) chlorhexidine, with levels of \textit{A. viscosus} T14AV again being below the detection limit. \textit{S. oralis} 34 was significantly reduced by 10 and 50 \( \mu g/ml \) chlorhexidine, with the reduction caused by 50 \( \mu g/ml \) chlorhexidine being significantly more than that of 10 \( \mu g/ml \) chlorhexidine. After 72 hours \textit{S. mutans} 10449 was significantly decreased by 10 and 50 \( \mu g/ml \) chlorhexidine, \textit{A. viscosus} T14AV was still significantly reduced by 50 \( \mu g/ml \) chlorhexidine to below the detection limit, and no significant differences could be seen in the levels of \textit{S. oralis} 34. Biofilm formation of the biofilms exposed to 10 and 50 \( \mu g/ml \) chlorhexidine was significantly reduced compared to the control biofilm at all time points measured (Figure 5.12). No significant difference could be seen between the different concentrations of chlorhexidine. The pH of the control biofilms was significantly lower than that of the chlorhexidine treated biofilms at all time points (Figure 5.13).

\textbf{5.12 Addition of mouthwash to the biofilm model}

There are many commercially available mouthwashes. Listerine and Savacol antiseptic mouthwashes were added to the biofilm model to compare their effectiveness with zoocin A and lauricidin. The addition of 1\% Listerine antiseptic mouthwash, or a matched ethanol control, to the biofilm model had no effect upon the bacterial count of any species present (Figure 5.14). One percent Savacol antiseptic mouthwash reduced the levels of \textit{S. mutans} 10449 by three logs and reduced \textit{S. oralis} 34 to below detectable levels. \textit{A. viscosus} T14AV was not significantly affected by the presence of 1\% Savacol antiseptic mouthwash. The pH of biofilms exposed to Savacol antiseptic mouthwash was 7.8. This was significantly higher than control biofilms, and those exposed to Listerine antiseptic mouthwash or a matched ethanol control, where the pH ranged from 5.1 - 5.7 (Figure 5.15). No difference could be seen between the pH of biofilms exposed to Listerine antiseptic mouthwash and the controls. The fluorescence of labelled biofilm cells was reduced by 40\% when biofilms were exposed to 1\% Listerine antiseptic mouthwash, compared to the control and ethanol control biofilms (Figure 5.16). Biofilms exposed to 1\% Savacol antiseptic mouthwash had 80\% less fluorescence, which was significantly lower than the control and ethanol control biofilms.
Figure 5.12: Fluorescence of FITC-WGA labelled biofilms cells exposed to chlorhexidine. Fluorescence over time where • is the control biofilm, ■ is a biofilm exposed to 10 µg/ml chlorhexidine and ▲ is a biofilm exposed to 50 µg/ml chlorhexidine.
Figure 5.13: The pH of biofilm cells exposed to chlorhexidine over 72 hours. pH of the biofilm cells where □ is the control, ■ is exposure to 10 µg/ml chlorhexidine and ● is exposure to 50 µg/ml chlorhexidine.
Figure 5.14: Exposure of biofilms to mouthwash. Bacterial count of the biofilms after 24 hours where ■ is the control, □ is exposure to a matched ethanol control, ☐ is exposure to 1% Listerine FreshBurst antiseptic mouthwash, and □□□□ is exposure to 1% Savacol antiseptic mouthwash.
Figure 5.15: The pH of biofilms exposed to mouthwash. pH of cells with varied biofilm treatments.
Figure 5.16: Fluorescence of FITC-WGA labelled biofilms cells exposed to mouthwash.
5.13 Resistance formation following 72 hours of antimicrobial exposure

The development of resistance is a major concern for any new antimicrobial. The MIC of the antimicrobials was determined before and after antimicrobial exposure in a 72 hour biofilm to test for resistance development. The MIC on agar of zoocin A and lauricidin against *S. mutans* 10449, *S. oralis* 34 and *A. viscosus* T14AV prior to biofilm growth was determined. *S. mutans* 10449 had a MIC of 50 µg/ml zoocin A on agar, while *S. oralis* 34 and *A. viscosus* T14AV were resistant to zoocin A (Table 5.1). All three species had a MIC for lauricidin of 60 µg/ml on agar. Following biofilm growth a single *S. mutans* 10449 colony, from the biofilm exposed to lauricidin, demonstrated increased resistance to lauricidin, showing growth on agar containing 180 µg/ml lauricidin. No other colonies exhibited antimicrobial resistance of greater than twice the original MIC. The *S. mutans* 10449 colony with increased lauricidin resistance (designated colony A) was taken for further investigation.

5.14 Further investigation of a colony displaying increased lauricidin resistance

To further investigate the *S. mutans* 10449 colony A, the MIC of lauricidin and zoocin A of the colony was determined in broth. When subcultured in BHI, colony A grew in BHI with a maximum lauricidin concentration of 5 µg/ml. This 5 µg/ml lauricidin broth culture and the colony A grown in BHI with no lauricidin were used to determine the MIC of zoocin A and lauricidin against the resistant colony and this was compared to the MIC for the original *S. mutans* strain. The MIC of lauricidin of the original *S. mutans* 10449 that had not been exposed to lauricidin in a biofilm was 15.63 µg/ml (Figure 5.17). The MIC of colony A subcultured in BHI was also 15.63 µg/ml. When colony A was subcultured in 5 µg/ml lauricidin the MIC was 31.25 µg/ml. The MIC of zoocin A for the original *S. mutans* 10449 was 6.25 µg/ml (Figure 5.18). The MIC of colony A subcultured in BHI was also 6.25 µg/ml, however growth can be seen starting after 20 hours of incubation in wells with *S. mutans* 10449 colony A with 6.25 µg/ml zoocin A. When colony A was subcultured in 5 µg/ml lauricidin the MIC was 12.5 µg/ml zoocin A.

When colony A was stabbed into CAB or 180 µg/ml lauricidin agar, growth was seen after incubation. Colony A also produced growth when struck onto CAB agar or used to inoculate a BHI broth, however, no growth was seen when it was struck onto 180 µg/ml lauricidin agar.
Table 5.1: Growth on antimicrobial containing agar following biofilm growth for 72 hours

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<tr>
<th>Bacterial species</th>
<th>Antimicrobial present in agar</th>
<th>Concentration of antimicrobial in agar (µg/ml)</th>
<th>No biofilm (initial MIC)</th>
<th>Control biofilm</th>
<th>Biofilm with zoocin A</th>
<th>Biofilm with lauricidin</th>
<th>Biofilm with zoocin A and lauricidin</th>
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Table 5.1: continued

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<th>Concentration of antimicrobial in agar (µg/ml)</th>
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<th>Biofilm with Zoocin A</th>
<th>Control biofilm</th>
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Number of stabs positive for growth from colonies (50) replica plated after recovery from biofilm growth.
Table 5.1: continued

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<th>Bacterial species</th>
<th>Antimicrobial present in agar</th>
<th>Concentration of antimicrobial in agar (µg/ml)</th>
<th>Number of stabs positive for growth from colonies (50) replica plated after recovery from biofilm growth:</th>
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<td>No biofilm (initial MIC)</td>
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Figure 5.17: Growth of colony A and wild type *S. mutans* with lauricidin. Growth of *S. mutans* where — is *S. mutans* alone; — is BHI alone; — is BHI with 125 μg/ml lauricidin; — is *S. mutans* with an ethanol control; and —, —, —, —, —, —, —, — and — are *S. mutans* with 125, 62.5, 31.25, 15.63, 7.81, 3.90, 1.95, 0.98 and 0.49 μg/ml lauricidin respectively. A is *S. mutans* colony A subcultured in BHI with 5 μg/ml lauricidin, B is *S. mutans* colony A subcultured in BHI and C is wild type *S. mutans*. 

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**Figure 5.17:** Growth of colony A and wild type *S. mutans* with lauricidin. Growth of *S. mutans* where — is *S. mutans* alone; — is BHI alone; — is BHI with 125 μg/ml lauricidin; — is *S. mutans* with an ethanol control; and —, —, —, —, —, —, —, — and — are *S. mutans* with 125, 62.5, 31.25, 15.63, 7.81, 3.90, 1.95, 0.98 and 0.49 μg/ml lauricidin respectively. A is *S. mutans* colony A subcultured in BHI with 5 μg/ml lauricidin, B is *S. mutans* colony A subcultured in BHI and C is wild type *S. mutans*. 

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Figure 5.18: Growth of colony A and wild type *S. mutans* with zoocin A. Growth of *S. mutans* where  is *S. mutans* alone;  is BHI alone;  is BHI with 100 μg/ml zoocin A;  is *S. mutans* with an elution buffer control; and , , , , , , , , and  are *S. mutans* with 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.2 and 0.1 μg/ml zoocin A respectively. A is *S. mutans* colony A subcultured in BHI with 5 μg/ml lauricidin, B is *S. mutans* colony A subcultured in BHI, and C is wild type *S. mutans*. 
When colony A was stabbed back into 180 μg/ml lauricidin agar from stabs into CAB and 180 μg/ml lauricidin agar, the streak on CAB, or from the BHI broth culture, growth was seen from the stab colonies in CAB and 180 μg/ml lauricidin agar, but not from the colony struck onto CAB or the broth culture.

5.15 Conclusions

In this chapter the biofilm model developed in chapter four was used to investigate the effects of zoocin A, lauricidin and both in combination on the biofilm. First the MIC of each antimicrobial against each bacterial species was determined to give an indication of existing antimicrobial resistance of the species. As expected *S. mutans* 10449 had a low MIC for zoocin A, while *S. oralis* 34 and *A. viscosus* T14AV were found to be resistant. All three species were found to be susceptible to lauricidin, with *S. oralis* 34 and *A. viscosus* T14AV being slightly more susceptible than *S. mutans* 10449.

It was found that zoocin A added to a biofilm incubated for 24 hours specifically targeted *S. mutans* 10449 and also resulted in an increase in *S. oralis* 34, while *A. viscosus* T14AV was unaffected. The addition of lauricidin to the biofilm model was able to reduce the levels of all three species at the higher concentrations tested (15 μg/ml and above), while 10 μg/ml lauricidin significantly reduced *S. mutans* 10449 alone. The use of both zoocin A and lauricidin in combination resulted in a significant increase in the reduction of *S. mutans* 10449, compared to the control biofilms or those exposed to the antimicrobials individually.

When the biofilm model was exposed to the antimicrobials for a longer period of time, *S. mutans* 10449 continued to be reduced by the antimicrobials. In contrast, *S. oralis* 34 and *A. viscosus* T14AV increased, especially in the presence of zoocin A or zoocin A with lauricidin. This increase of bacterial numbers in the presence of the antimicrobials may be due to reduced competition by *S. mutans* 10449. It was found that the addition of zoocin A and lauricidin in combination prevented the reduction of pH seen in the control biofilms, whereas the addition of zoocin A and lauricidin separately had a reduced pH compared to the combination. However, the pH of biofilms exposed to either antimicrobial individually was still significantly higher than that of the control biofilms. It was shown that *S. mutans* 10449 cells growing in the presence of sucrose (growing in a biofilm) had significantly higher fluorescence per cell when labelled with FITC-WGA. This confirmed that FITC-WGA could
be used as a measure of biofilm formation. Labelling biofilm cells with FITC-WGA gave significantly less fluorescence when biofilms were exposed to the antimicrobials compared to the control biofilms, showing biofilm formation was reduced. Examination of the biofilms with scanning electron microscopy also demonstrated that biofilm formation was reduced in the presence of the antimicrobials, especially when zoocin A and lauricidin were used in combination.

Chlorhexidine was added to the biofilms as it is has been investigated extensively as an antimicrobial for the oral cavity and therefore could be used to benchmark the effectiveness of zoocin A and lauricidin. Chlorhexidine resulted in a substantial reduction of all three species at all three time points, especially at a concentration of 50 μg/ml. Biofilm formation was also reduced by chlorhexidine at both 10 and 50 μg/ml, and the decrease in pH seen with the control biofilm was prevented. Savacol antiseptic mouthwash and Listerine FreshBurst antiseptic mouthwash are two commercially available mouthwashes used to reduce dental plaque. They were added at a 1% concentration so their active ingredients were at a similar molar concentration to zoocin A and lauricidin. Listerine FreshBurst antiseptic mouthwash contains a high proportion of alcohol (22%) so a matched ethanol control was used. Listerine FreshBurst antiseptic mouthwash at 1% did not have a detectable effect upon the biofilm cells and nor did the ethanol control. The cfu was not reduced compared to the control, the pH of the biofilm cells was similar to that of the control and fluorescence was not significantly reduced when cells were labelled with FITC-WGA. Savacol antiseptic mouthwash at 1% resulted in decreased levels of *S. mutans* 10449 and *S. oralis* 34 and prevented any decrease in pH of the biofilms cells. When fluorescently labelled, the biofilm fluorescence was also found to be significantly reduced. Unlike zoocin A in combination with lauricidin, chlorhexidine and Savacol antiseptic mouthwash reduced the bacterial count of all three species.

With any new antimicrobial treatment it is important to consider the development of bacterial resistance. When exposed to zoocin A, lauricidin or both in combination for 72 hours, only one colony with increased antimicrobial resistance was seen. This colony arose from the biofilms exposed to lauricidin and was found to have slightly increased resistance to both lauricidin and zoocin A. Using antimicrobials in combination is proposed as a way to reduce the development of resistance\textsuperscript{52} and no resistance was seen in biofilms exposed to zoocin A and lauricidin in combination.
6. Discussion

6.1 Production of zoocin A

6.1.1 Quantification of protein

6.1.1.1 Measuring the concentration of zoocin A

To use zoocin A as an antimicrobial it is important to be able to accurately quantify the protein. Accurate quantification is required for meaningful analysis of the protein yield during recombinant protein production and purification\textsuperscript{394} and for consistency in experiments using zoocin A. Spectrophotometric assays are simple, sensitive, high-throughput assays and are frequently used for protein quantification.\textsuperscript{325} The Bradford assay is a frequently used spectrophotometric assay and was therefore investigated as a method to quantify zoocin A.

The Bradford assay utilises the change in absorbance spectra of Coomassie brilliant blue stain when it binds to protein\textsuperscript{55} and the dye changes from an absorption maximum at 465 to 595 nm.\textsuperscript{55} The dye-protein complex has a high extinction coefficient, making the assay very sensitive.\textsuperscript{55} The Bradford assay has previously been shown to be very reproducible.\textsuperscript{55,325,394} However, in this study three separate Bradford assays on the same zoocin A sample gave very different results (17,288 µg/ml ± 22,354), indicating the Bradford assay is not suitable for the quantification of zoocin A. Several factors can impact upon the accuracy of the Bradford assay. The relationship between absorbance and protein concentration is not linear, as there is an overlap in the absorbance spectra of the two colour forms of the dye. This means the background absorption of the reagent decreases as more dye is bound to protein.\textsuperscript{55} Bradford\textsuperscript{55} used protein concentrations up to 100 µg/ml and noted that unknown concentrations could still be calculated as long as the assay was run with a set of standards and the unknown sample concentration was determined using the response curve of the protein standards. Simonian and Smith\textsuperscript{394} suggest the Bradford is best used for protein concentrations of up to 10 µg/ml (the micro Bradford assay), as this is most sensitive,\textsuperscript{325} but notes the Bradford assay is easily adapted for up to 100 µg/ml of protein by increasing the concentration of dye in the reaction. In this study the Bradford assays were carried out with BSA standards ranging from 0 to 15.62 µg/ml, which is within the range of accuracy for the Bradford assay. When constructing the standard curve, the $r^2$ value was always above 0.99,
indicating the data were very close to being linear and that the nonlinearity of the standard curve was unlikely to be the cause of variation between Bradford assays. In addition, the unknown zoocin A sample was diluted so the absorbance of the sample was always within the limits of the standard curve. The colourimetric dye Coomassie brilliant blue has a higher affinity for some amino acids, which leads to variation in how different proteins react to the dye. Although differences between binding of Coomassie brilliant blue to the BSA standard and zoocin A could reduce the accuracy of the assay, this would be expected to result in a consistent over- or under-estimation of the actual protein concentration, rather than the large variation in protein concentration estimates for the same sample that were seen in this study. The Bradford assay can also be affected by substances in the protein buffer such as glycerol, detergents, β-mercaptoethanol, acetic acid, ammonium sulfate, tris, and some alkaline buffers. Alkaline buffers result in slightly more colour development during the assay. The zoocin A buffer (elution buffer) was alkaline, which may explain the large variation in the assay results, although the zoocin A sample buffer was at the same concentration each time. Akesson et al. used the Bradford assay and a BSA standard to quantify zoocin A, but it is possible different buffers were used.

A colourimetric assay developed by Itzhaki and Gill was used as an alternative spectrophotometric assay to the Bradford assay. The Itzhaki assay is based upon measuring the absorbance of the protein and copper complex formed in alkaline copper sulphate solutions. The interaction between copper and protein is based on a reaction between the copper and the peptide bonds of the protein. This means differences in protein composition have a minimal effect on the assay results. The Itzhaki assay gave very consistent results (5122 µg/ml ± 92) when used to repeatedly assay the same zoocin A sample and was therefore used to quantify zoocin A throughout this study. Although the Itzhaki assay is not as sensitive as the Bradford assay, it gives a linear standard curve with up to 530 µg/ml of protein. This simplifies the assay, as protein samples do not need to be diluted into a narrow assay range. Unlike the Bradford assay, the Itzhaki assay is not affected by alkaline protein buffers, as it is carried out under alkaline conditions, and is therefore unlikely to be affected by the zoocin A buffer.

Adoption of the Itzhaki assay assured experimental reproducibility. However, both the Bradford and Itzhaki assays used a set of BSA standards. To obtain the most accurate result the standard for a protein assay should be the same as the unknown protein sample, where the concentration of the standard has been calculated using a higher-order method.
avoids any inaccuracies resulting from the assay reagent reacting differently with proteins of different composition. To achieve an accurate protein standard zoocin A was dialysed, lyophilised and resuspended at a known concentration by weight. Spot tests before lyophilisation and after resuspension indicated that zoocin A activity was not lost during this process.

6.1.1.2 Measuring zoocin A activity

The Itzhaki assay measures the protein concentration. However, it is also important to measure the activity of zoocin A samples, as the presence of zoocin A protein is not a guarantee of its activity and its activity is crucial for the use of zoocin A as an antimicrobial. Spectrophotometric assays require pure protein samples, so measurement of zoocin A activity also allows the amount of zoocin A in unpurified samples to be estimated. Simmonds et al.\(^\text{390}\) used the spot test to determine the activity of zoocin A. Activity was measured in AU/ml. The spot test is a simple method to assay zoocin A activity, but it is only accurate within a factor of two and requires overnight incubation. The DRA was therefore investigated as an alternative activity assay. Zhou et al.\(^\text{501}\) developed a DRA for determining the effectiveness of lysostaphin, an enzyme which also cleaves peptidoglycan.\(^\text{62}\) The assay utilised the lysostaphin substrate, either whole cells or peptidoglycan, dyed with remazol brilliant blue. This DLS was stable and did not leak dye.\(^\text{501}\) Upon cleavage by lysostaphin, the substrate released a soluble dye product and the change in absorbance was recorded after the removal of excess dye labelled substrate.\(^\text{501}\) Lai et al.\(^\text{247}\) used the DRA to determine the activity of zoocin A samples using dye labelled whole cells of various target species. Dye labelled \textit{S. pyogenes} FF22 with an OD_{595} of 1.5 was used as the positive control for dye release. The DRA was sensitive and reproducible.\(^\text{247}\) A modified version of this assay was developed to assay zoocin A activity in this study. Zhou et al.\(^\text{501}\) measured activity in active units, where one unit was defined as a change in absorbance of 0.01 per minute per centimetre of path length, whereas Lai et al.\(^\text{247}\) measured activity in arbitrary units of dye release per minute per mM of zoocin A. To give consistency between the results of the spot test and DRA, activity in the dye release assay was measured against a standard curve of zoocin A activity measured in AU/ml. The release of dye was linear only at low concentrations, up to 10 AU/ml of zoocin A. Above 10 AU/ml of zoocin A dye release plateaued, suggesting the assay was substrate limited. Zhou et al.\(^\text{501}\) found dye release was linear at protein concentrations between 0.25 and 2 µg/ml and was directly proportional to enzyme concentration when reactions were incubated for 20 minutes.\(^\text{501}\) Lai et al.\(^\text{247}\) also found the rate of dye release was linear over time and was proportional to DLS and enzyme
concentration. However, long incubation times of over 30 minutes gave nonlinear responses. In this study an initial DRA was incubated for 30 minutes, and dye release at low protein concentrations did not always produce consistent standard curves. Therefore the effect of assay incubation time was investigated. If substrate limitation was a problem a shorter incubation time would be expected to improve the linearity of the standard curve. A shorter incubation time resulted in a more linear standard curve with a higher $r^2$ value and an incubation time of 10 minutes was used in all future DRA.

Zhou et al.\textsuperscript{501} and Lai et al.\textsuperscript{247} used different concentrations of DLS. The effect of DLS concentration upon the DRA was therefore investigated to determine the optimal concentration. No obvious relationship between the linearity of dye release and DLS concentration was seen. If the assay was still substrate limited at the low zoocin A concentrations used, it would be expected that higher concentrations of DLS would result in a more linear standard curve. However, the standard curve with DLS with an OD$_{595}$ of 3.0 had the lowest $r^2$ value. This may be because the concentration of DLS is too high for low concentrations of zoocin A to cleave the DLS a sufficient number of times to release a soluble dye product in a linear fashion within the 10 minute assay. As the $r^2$ value was highest, and above 0.99 with DLS with an OD$_{595}$ of 2.0, this concentration of DLS was used in future dye release assays.

It was difficult to accurately dilute samples with a high concentration of zoocin A to the limited range of the assay (2–10 AU/ml) and for these samples several different dilutions were needed to ensure one would be in the required concentration range. This increased the number of samples to be processed. However, it was found that the DRA was not suitable for processing a large number of zoocin A samples in a single assay, as the time required to add the DLS to the samples and to halt the reaction by the addition of 95% ethanol made it difficult to keep to a standard incubation time of 10 minutes for all samples. For these reasons the DRA was used to more accurately quantify samples with a low concentration of zoocin A and the spot test was used to quantify concentrated zoocin A samples and to ensure the activity of purified zoocin A correlated with concentration of zoocin A determined by the Itzhaki assay.
6.1.2 Production of zoocin A

6.1.2.1 Zoocin A producer strains
The amount of zoocin A that could be produced was investigated, as it needed to be produced at sufficient concentrations to use zoocin A as an antimicrobial agent in a biofilm model. The production of zoocin A from *S. equi* subspecies *zooepidemicus* 4881 in a static flask and recombinant *E. coli* grown in both 500 ml shake flasks and 10 L volumes in a fermentor was compared. The amount of zoocin A produced was measured in AU as this allowed the quantification of zoocin A in unpurified protein samples.

Simmonds et al.\(^{390}\) found *S. equi* subspecies *zooepidemicus* 4881 produced 2 AU/ml zoocin A in BHI (2000 AU per litre of culture), although supplementation of a chemically defined medium with 0.5% lactose, sorbitol, ribose, or maltose improved the yield. A defined medium supplemented with 0.5% maltose resulted in 16 AU/ml zoocin A, which was achieved after 12 to 16 hours of incubation. Maximum yield was achieved with pH-controlled chemically defined media, where titres of 64 AU/ml were achieved. In this study growth of *S. equi* subspecies *zooepidemicus* 4881 in a static flask of BHI produced 3000 AU per litre of culture, which is similar to the production seen by Simmonds et al.\(^{390}\) However, the culture was incubated for only 9.5 hours (four hours after an OD\(_{595}\) of 0.7 was reached) in order to be comparable to the incubation period of the recombinant *E. coli* strain, and this incubation period may not have been sufficient to achieve maximum zoocin A production.

Lai et al.\(^{247}\) developed a recombinant *E. coli* strain to produce zoocin A. The recombinant zoocin A had the same spectrum of biological activity as native zoocin A. Lai et al.\(^{247}\) purified 1.523 mg of zoocin A from 50 ml cultures. This is equivalent to 152 mg per litre of culture, which is very similar to the titres achieved in this study, where growth of the recombinant *E. coli* strain\(^ {247}\) in 500 ml shake flasks produced 192 mg (98,304 AU) per litre of culture after protein purification. This is over 30 times the protein produced with a static culture of *S. equi* subspecies *zooepidemicus* 4881 grown in BHI in this study, and 1.5 times the amount of protein produced by Simmonds et al.\(^ {390}\) using a chemically defined medium with pH control. This increase can be attributed to the inducible nature of the zoocin A gene in the recombinant *E. coli* strain and its rapid growth compared to *S. equi* subspecies *zooepidemicus* 4881. Induction of a recombinant gene leads to up to 30% of protein production being devoted to recombinant protein production.\(^ {290}\)
To produce the amount of zoocin A required for this study production was scaled up to 10 L in a benchtop fermentor. Scaling up culture volumes can result in reduced mixing efficiency leading to chemical gradients in the culture media and inducing stress responses in the cells.\textsuperscript{167,321} This can ultimately result in reduced protein yield.\textsuperscript{379,488} Growth of the recombinant \textit{E. coli} strain in a 10 L culture was slightly slower than in 500 ml shake flasks, but the final OD was not changed. This reduction in growth rate may be due to limited oxygen in the media. Compressed air was sparged at 10 standard litres per minute, the maximum rate the fermentor could maintain. However, Liu et al.\textsuperscript{271} found that for prolonged exponential growth sufficient oxygen could not be supplied with air alone and that pure oxygen was required. Despite possible limitations on the growth of \textit{E. coli} in the fermentor the culture produced 165 mg (81,920 AU) of zoocin A per litre of culture, which is similar to the amount produced in shake flasks. Therefore, the scaling up of the culture did not reduce the overall protein yield.

The yield of 165 mg per litre of culture of zoocin A is higher than that achieved by several studies producing recombinant protein for structural analysis. Leon et al.\textsuperscript{252} produced 32 mg per litre of culture of haemoglobin I from \textit{Lucina pectinata}, while Dieci et al.\textsuperscript{108} used an \textit{E. coli} strain overexpressing \textit{argU} to produce five recombinant proteins, achieving yields of 10 to 50 mg per litre of culture. However, all these proteins were from eukaryotic sources, which may result in decreased yield. Mosbah et al.\textsuperscript{312} produced 5.3 mg of recombinant \textit{Staphylococcus xylosus} extracellular lipase in 50 ml \textit{E. coli} cultures, which equates to 530 mg per litre of culture, considerably more than was achieved in this study.

\subsection*{6.1.2.2 Recombinant zoocin A production}

Previous studies producing recombinant zoocin A have incubated the culture for five hours following induction of the culture with IPTG.\textsuperscript{247} It was found more rapid production of zoocin A could be achieved by reducing this incubation time, as most zoocin A was produced within the first hour of incubation after induction. Zoocin A production was measured in AU/ml to allow the quantification of unpurified zoocin A samples. Three hours after induction the amount of zoocin A produced represented 98\% of that produced after five hours. Maximum zoocin A production was reached by four hours and the increase of zoocin A in the final hour was less than 1\%. Further experiments for the production of zoocin A were therefore incubated for four hours following induction.
Induction of recombinant systems with IPTG can reduce the final biomass of the culture and induce stress response proteins that can reduce protein yield. While previous studies had used a final concentration of 1 mM IPTG to produce recombinant zoocin A, it was found that 0.1 mM IPTG was sufficient to induce maximum zoocin A production after four hours of incubation. This study found no difference in the growth rate of *E. coli* induced with different concentrations of IPTG. In contrast, Aggarwal et al. found growth of *E. coli* was slightly reduced with 1 mM IPTG when compared to 0.1 mM IPTG. Other studies involving recombinant protein in *E. coli* have also used 0.1 mM IPTG and using ten times less IPTG reduces the cost of zoocin A production, especially when inducing a 10 L culture.

### 6.1.2.3 Optimising codon usage

Rare codons in a recombinant protein sequence can reduce the final protein yield achieved, so the zoocin A gene sequence was analysed for the occurrence of rare codons. Rare codons are often considered to be codons that are used at a frequency of less than 1% of all codons. However, this definition also includes codons which are used more frequently for a particular amino acid, but the amino acid encoded is used infrequently. Codon usage can also be determined by finding the preferred codon for each amino acid. By comparing the abundance of a particular codon to that of all other codons for the same amino acid, the codon preference for a particular amino acid can be established. Five codons in the zoocin A gene were identified as being infrequently used for a particular amino acid and all also occurred with a frequency of less than 1% in *E. coli*. Of these rare codons AGA was identified as most likely to interfere with protein synthesis. Brinkmann et al. found expression of proteins from genes containing numerous AGA or AGG codons (both recognised by the same tRNA) could be improved by providing the *dnaY* (*argU*) gene, encoding this tRNA, on a plasmid. Chen and Inouye found that the AGA and AGG arginine codons are used preferentially within the first 25 codons of *E. coli* genes and suggest that this is involved in regulation of gene expression. A cluster of AGA codons inserted at the beginning of the *lacZ* gene almost completely inhibited expression of the gene in a model system. This inhibition was reduced by co-expression of the *argU* gene, or by moving the AGA cluster downstream to more than 50 codons away from the initiation codon. The zoocin A gene has an AGA codon as the second codon, which has the potential to impact upon gene expression and therefore codon optimisation was carried out.

Two approaches to codon optimisation were used. The first AGA codon in the zoocin A gene was identified as having the potential to reduced gene expression, and this codon was
altered by site-directed mutagenesis to CGT, a codon more commonly used in *E. coli*. Site-directed mutagenesis is a relatively simple and reliable method to make small changes to the gene sequence\(^1\) and has previously been used to alter AGA codons in a recombinant gene expressed in *E. coli*.\(^6\) The second approach utilised a codon plus plasmid encoding three tRNAs that recognise rare *E. coli* codons. Plasmids encoding tRNA molecules corresponding to rare codons have previously been used to alleviate problems encountered when expressing recombinant genes containing rare codons.\(^{24,108}\) This approach has the advantage of encoding tRNAs for three of the codons present in the zoocin A gene that were identified as being used with low frequency in *E. coli*. However, it also added a third plasmid to the zoocin A producer strain, which raised the possibility that the energy required to maintain all three plasmids may reduce the overall protein yield.\(^1\)

Alteration of the first AGA codon by site-directed mutagenesis did not increase zoocin A production. This suggests that the early AGA codon in the zoocin A gene is not reducing protein yield, possibly as the next AGA codons is not until the 46\(^{th}\) codon. Although studies have indicated that the presence of AGA codons at the beginning of the gene sequence is more likely to affect protein yield, these studies generally involve clusters of AGA codons.\(^{80,220}\) Clusters of rare codons have been noted in *E. coli* genes and have been suggested to play a role in protein expression control.\(^{86,345}\) Zoocin A yield was also not improved by insertion of the pQE-80L ZooA1 and pREP4 plasmids into *E. coli* BL21-CodonPlus-(DE3)-RIL (Stratagene). The codon plus plasmid encodes the tRNAs which recognise the AGA/AGG, AUA and CUA codons. This result confirms that the initial AGA codon in the zoocin A gene did not reduce protein yield. It also suggests the presence of AUA and CUA codons were also not reducing zoocin A production, probably because neither codon occurs with high frequency in the zoocin A gene and they do not occur in clusters. As protein production was not reduced, this result also suggests that the presence of a third plasmid did not impact upon protein yield. However, this experiment was carried out in a different strain of *E. coli* and it is possible that any improvement in yield due to improved codon usage was mitigated by the presence of a third plasmid. While the codon plus *E. coli* strain did not improve protein yield, it did reach an OD\(_{595}\) of 0.7 in approximately three hours, whereas *E. coli* M15 pQE-80L, pREP4 took four hours to reach the same OD and this meant zoocin A could be produced more rapidly. The rare codon GGA occurs 16 times in the zoocin A gene, it is the most common rare codon present. It is possible the frequency of this codon could reduce the recombinant protein yield. However, the GGA codon is not commonly identified in the literature as a problematic codon and it does not occur in clusters.
Kim and Lee\textsuperscript{220} found that clusters of rare codons, rather than the overall frequency of the rare codon, could limit recombinant gene expression. While many studies have improved protein yield in \textit{E. coli} by codon optimisation, these studies involve recombinant genes from archaea,\textsuperscript{219} protists\textsuperscript{24} and other eukaryotic sources.\textsuperscript{69,108} Few studies have used codon optimisation for expressing other bacterial genes in \textit{E. coli}\textsuperscript{18} and codon optimisation did not improve protein yield. This suggests that any codon bias present in the zoocin A gene is not sufficient to have a meaningful impact on protein yield and is therefore not a limiting factor in recombinant zoocin A production.

6.2 Development of a biofilm model

Biofilm models vary; they can contain a single species,\textsuperscript{14} several defined species,\textsuperscript{130} or involve oral microcosms grown from saliva samples.\textsuperscript{180,399} While a single species biofilm is the simplest, interactions between bacterial species can reduce antimicrobial susceptibility\textsuperscript{207,348} and change the cariogenic potential of the biofilm.\textsuperscript{225} This study used a triple-species biofilm model comprised of \textit{S. mutans} 10449, \textit{S. oralis} 34 and \textit{A. viscosus} T14AV to more accurately model bacterial interactions and the effects of specifically targeting \textit{S. mutans} on other bacteria in the biofilm. \textit{S. mutans} was included as a prime etiological agent of dental caries\textsuperscript{53,427,447} and a target of zoocin A.\textsuperscript{188,390} \textit{S. oralis} and \textit{A. viscosus} were included as common oral species\textsuperscript{2,121} that are primary biofilm colonisers\textsuperscript{231,254,432} and not susceptible to zoocin A.\textsuperscript{390,391} \textit{S. oralis} was also included as its peptidoglycan cannot be cleaved by zoocin A.\textsuperscript{391}

6.2.1 Enumeration of bacteria

To utilise a multi-species biofilm model each species must be enumerated individually. This can be achieved using selective media, which are often used for the isolation of a particular species from mixed cultures.\textsuperscript{322} These selective conditions avoid contamination or overgrowth by an unwanted species. However, they often also reduce the growth of the species being isolated,\textsuperscript{49,322} as the presence of subinhibitory concentrations of antimicrobials can restrict bacterial growth, especially when the inocula are small.\textsuperscript{316,343} It was therefore important to compare growth of the strains used in this study under selective and nonselective conditions. Previous studies have found selective conditions for the growth of oral bacteria can reduce the recovery of the desired bacteria. Emilson and Bratthall\textsuperscript{123} compared the growth of \textit{S. mutans} on agars selective for \textit{S. mutans} with growth on nonselective agars on
which *S. mutans* can be identified morphologically. It was found the cfu of *S. mutans* was reduced around 10% on the selective agars and that some serotypes of *S. mutans* were unable to grow on the selective media. Little et al.\textsuperscript{270} found the recovery of *S. mutans* varied on different selective media, that different *S. mutans* serotypes were inhibited by different selective conditions, and that media selective for *S. mutans* significantly reduced the cfu of *S. mutans* compared to nonselective agars. Similar findings were also described by Wan et al.\textsuperscript{462} in a more recent study. However, no significant difference in growth of the three species on selective and nonselective agars was seen in this study and no species grew on an agar designed to select for a different species. The selective conditions did not reduce the viability of each species and were therefore suitable for the enumeration of individual species from the biofilm model.

### 6.2.2 Biofilm media

Biofilm models use a variety of different nutrient media, often based on the complexity of the model and thus its ability to mimic the oral environment. Simple biofilm models frequently use dilute nutrient media,\textsuperscript{78,458} often supplemented with sucrose,\textsuperscript{240} while more complex models use artificial saliva\textsuperscript{96,180} or sterilised whole human saliva.\textsuperscript{133} To keep the biofilm model simple and practical, a nutrient broth supplemented with 1% sucrose was chosen as the nutrient medium. While some studies have used TSB\textsuperscript{313} or Schaedler broth\textsuperscript{130} as nutrient broths for biofilms models, THB\textsuperscript{12,500} and BHI\textsuperscript{14,367} are more commonly used and were therefore compared as the nutrient source for the triple-species biofilm. Aldsworth et al.\textsuperscript{12} used THB to grow a biofilm of *S. mutans*, *A. viscosus* and *L. casei*. However, in this study THB at 1/3 strength did not support the growth of *A. viscosus* T14AV and biofilms grown in THB had greater variation than those in BHI. Therefore THB was not suitable as a nutrient source for the biofilm model in this study. The bacterial count of biofilms grown with 1/5 strength BHI was reduced compared to the bacterial inoculum used and described in section 4.5.1. However, biofilms grown in 1/3 strength BHI had increased levels of *S. mutans* 10449 and *S. oralis* 34 compared to the initial inoculum and 1/3 strength BHI supplemented with 1% sucrose was therefore selected as the nutrient medium for the biofilm model.

### 6.2.3 Bead conditioning

Most biofilm models coat the substrate in filtered human saliva\textsuperscript{149,313} or an artificial saliva, to mimic the formation of the acquired pellicle.\textsuperscript{130,367} Artificial saliva formulations usually
contain mucin, which has been shown to coat glass surfaces within five minutes and forms a pellicle to which the bacteria can attach. A mucin-containing BMM was therefore used to condition the glass beads used as a biofilm substrate in this study.

The method used to coat the substrate and inoculate the biofilm model varies between biofilm models. Artificial saliva can be allowed to coat the biofilm substratum for five minutes to 24 hours, with several studies allowing two to four hours. Some models then allow the artificial saliva to dry before bacterial inoculation. In dynamic biofilm models the bacterial inoculum is often allowed to attach before the nutrient flow is begun. This attachment period can range from 15 to 30 minutes prior to the nutrient flow beginning. A range of methods were therefore trialled to determine the optimal bead conditioning and inoculation method. The bacterial count of the conditioned and inoculated beads under varied bead conditioning methods was compared with those incubated for 24 hours to form a biofilm.

All methods involved the same bacterial inoculation and resulted in similar levels of bacteria on the beads after conditioning. However, some small differences could be seen. In particular method C, which involved overnight incubation, resulted in the highest level of bacteria. When biofilm growth after 24 hours was determined for each bead conditioning method substantial differences between the methods became apparent. Bead conditioning methods D and E were not suitable as they resulted in the reduced growth of some bacterial species in the biofilm. In particular method D (where the beads were uncoated) did not support the growth of S. oralis and the levels of A. viscosus T14AV were significantly reduced, which indicates that coating of the beads is required for the attachment or growth of these species. This is probably because salivary components of the acquired pellicle act as specific receptors for the binding of primary colonisers to the biofilm substrate. Gibbons et al. found adhesion of S. sanguis to salivary coated surfaces was mediated by bacterial recognition of sialic acid, found in salivary components such as mucin, and that adhesion was reduced when the saliva coated surface was treated with an enzyme which cleaved sialic acid linkages. The use of artificial saliva to coat the biofilm substrate is therefore required to provide receptors to which the bacteria can attach. Methods B and D, which involved dried beads, were also not suitable, as they gave more variable results when compared to methods A and C, where the beads were still covered in BMM when they were inoculated. Methods A and C resulted in very similar and reproducible biofilms after 24 hours of incubation, despite the beads conditioned by method C having significantly more bacteria prior to biofilm growth.
than those conditioned by method A. The growth of *S. mutans* 10449 and *S. oralis* 34 in the first 24 hours was therefore greater with method A. Method A was used in all subsequent biofilm experiments as it gave reproducible growth of all three species in the biofilm and bacterial growth over 24 hours of incubation was greater than that seen with method C.

### 6.2.4 Sonication of bacteria

Sonication is frequently used to remove bacterial cells from a biofilm substrate and is also used to disperse the biofilm cells before enumeration. The ability of sonication to remove biofilm cells from the glass bead substrate was therefore tested.

This was initially examined by determining the number of bacterial cells removed by subsequent sonication steps. Each sonication reduced the bacterial count of each species by at least one log and after three sonication steps the amount of *A. viscosus* T14AV removed by further sonication steps was below the detection limit. This suggests the initial three sonication steps removed the majority of the bacterial cells. This is supported by studies which have used sonication to dislodge biofilm cells. Li et al. found sonication removed over 99% of cells from *S. mutans* biofilms grown on microtitre plates and Williams et al. found sonication and vortexing of *S. aureus* biofilms removed over 95% of biofilm cells. However, it is possible that a population of cells could not be removed by sonication and remained attached to the glass beads, which would mean the bacterial cells being removed from the beads was not necessarily representative of the bacterial population in the biofilm. Lindsay and von Holy found that using vortexing or sonication to remove *Pseudomonas fluorescens* or *B. subtilis* biofilms from stainless steel or polyurethane substrates left residual EPS and attached cells, while shaking with beads removed all bacterial cells. Scanning electron microscopy was used to confirm that no visible bacterial growth was present on both unused beads and those where the biofilm had been removed by sonication. Therefore sonication was a suitable method for removing biofilm growth from the substrate in this study.

The effect of sonication on the bacterial cfu was investigated to ensure the sonication process did not reduce bacterial viability. Since the sonication process is required to remove the bacterial biofilm from the glass beads, it was not possible to investigate the effect of sonication on biofilm replicates and have an appropriate sonication-free control. Therefore the effect of sonication upon bacterial cells grown in a broth culture was tested. To mimic
the biofilm harvesting process the bacteria were added to sintered glass cups containing glass beads, with PBS added. No difference in the bacterial count was seen, indicating the sonication process did not reduce the viability of the bacterial cells. Many studies use sonication to dislodge or disperse cells and several have also shown sonication does not reduce cell viability. Soukka et al.\textsuperscript{407} found sonication did not reduce the viability of planktonic \textit{S. mutans}, while Vickerman et al.\textsuperscript{459} found sonication did not reduce the viability of \textit{S. gordonii} biofilm cells. To ensure these results were applicable to biofilm cells in this model, three biofilms were grown for 24 hours and biofilm cells were removed from the beads as much as possible by pipetting. Each biofilm was then divided in two, so that each sonicated biofilm had a sonication-free control. Again, no reduction in viability was seen when cells were sonicated, in fact significantly more \textit{S. mutans} cells were recovered in the sonicated samples. Biofilm cells form aggregates\textsuperscript{230,231} and therefore the increase in cfu seen for \textit{S. mutans} with sonication is likely to be due to the sonication process dispersing cell clumps in the biofilm cells.

\textbf{6.2.5 Biofilm growth over time}

The growth of the biofilm over time was investigated to determine suitable sampling times for the model. \textit{S. mutans}, \textit{S. oralis} and \textit{A. viscosus} are all primary colonisers of the oral biofilm; however, \textit{S. mutans} 10449 and \textit{S. oralis} 34 both initially increased in number, while \textit{A. viscosus} T14AV levels decreased from the inoculation level and then remained reasonably stable. This decrease is likely to be due to \textit{A. viscosus} T14AV being unable to maintain the inoculation levels due to competition by the streptococci, especially as streptococci are the predominant early colonisers, \textit{S. oralis} in particular.\textsuperscript{231,254,328} The role of \textit{S. oralis} as an early coloniser also explains the initial increase in \textit{S. oralis} 34, over the first 36 to 48 hours of biofilm incubation, as \textit{S. oralis} can play a central role in biofilm development. These results are comparable to those seen by Li et al.\textsuperscript{254} who reported that streptococci, especially \textit{S. mitis} and \textit{S. oralis} increased during the first six hours of biofilm development, while \textit{Actinomyces} species remained constant as overall bacterial levels increased, decreasing the proportion of \textit{Actinomyces} species in the biofilm. While the bacterial count of \textit{S. mutans} 10449 and \textit{A. viscosus} T14AV remains constant after 36 hours of incubation, the levels of \textit{S. oralis} 34 initially increase and then decrease sharply after 60 hours of incubation. This is likely to be due to competition and acid production by \textit{S. mutans} 10449. The continuous flow of the sucrose-containing nutrient media will have led to constant production of lactic acid by the bacteria as they metabolise the sucrose. \textit{S. mutans} and other cariogenic bacteria are
particularly acid tolerant\textsuperscript{354,386} and can maintain glycolytic processes at low pH, which is achieved by maintaining a neutral internal pH.\textsuperscript{42,386} The energy to maintain the internal pH of the bacteria is provided by the continuation of glycolysis.\textsuperscript{386} This leads to continued acid production and survival by \textit{S. mutans}. As \textit{S. oralis} is less acid tolerant than \textit{S. mutans},\textsuperscript{426} this process could lead to the decline in \textit{S. oralis} towards the end of the biofilm incubation.

The biofilm model in this study was incubated for 72 hours. The length of incubation for other biofilm models varies. Studies on biofilm formation\textsuperscript{133,313} and some studies on the effectiveness of various antimicrobials\textsuperscript{14,78} are incubated for 14 to 24 hours. Other studies on antimicrobial effectiveness run for around 72 hours.\textsuperscript{208,383} A mature oral biofilm takes several days to develop\textsuperscript{10,231} and Kinniment et al.\textsuperscript{222} found a multispecies biofilm model involving both primary and secondary colonisers took 100 hours to reach a steady state. Some biofilm models are incubated for 10 to 30 days to examine long-term biofilm formation\textsuperscript{96,480} or biofilms in a steady state.\textsuperscript{106,180} However, this biofilm model only involves early colonisers and the investigation of whether antimicrobials can reduce the levels of \textit{S. mutans} in a developing biofilm and was therefore incubated for a maximum of 72 hours, with sampling done after 24, 48 and 72 hours of incubation.

\subsection*{6.3 Addition of antimicrobials to the biofilm model}

\subsubsection*{6.3.1 Minimum inhibitory concentration of zoocin A and lauricidin in broth}

The MIC of zoocin A and lauricidin was determined in order to establish the sensitivity of each bacterial species to the antimicrobials and give a starting point for the amount of each antimicrobial to add to the biofilm model. As expected, \textit{S. mutans} was very sensitive to zoocin A, while the growth of \textit{S. oralis} and \textit{A. viscosus} was unaffected. These results agree with previous studies, which found all \textit{A. viscosus} strains tested were resistant to zoocin A.\textsuperscript{390} While Simmonds et al.\textsuperscript{390} identified an \textit{S. oralis} strain which was sensitive to zoocin A, other studies have found all \textit{S. oralis} strains tested to be resistant.\textsuperscript{8,391} The MIC of zoocin A for \textit{S. mutans} 10449 was 6.25 µg/ml, while Akesson et al.\textsuperscript{8} reported the MIC of this strain to be 1 µg/ml. This small difference may be due to differences in the methodology, as Akesson et al.\textsuperscript{8} used the Bradford assay to quantify the zoocin A. All three bacterial species were found to be sensitive to lauricidin. \textit{S. mutans} 10449 was slightly more resistant to lauricidin, with an MIC of 25 µg/ml, as opposed to 12.5 µg/ml for \textit{S. oralis} 34 and \textit{A. viscosus} T14AV. These results are consistent with those previously reported: Kabara and Vrable\textsuperscript{203} reported the
MIC of monolaurin for *S. mutans* to be between 15.6 and 31.2 µg/ml, depending on pH, and that *S. mutans* was more sensitive to monolaurin as the pH decreased. The MIC of monolaurin for *A. viscosus* has been reported as 15.6 µg/ml and several other studies have shown the sensitivity of streptococci to monolaurin.

### 6.3.2 Addition of zoocin A to the biofilm model

Of the species present in the biofilm model, zoocin A specifically targets *S. mutans* and was therefore applied to reduce the proportion of *S. mutans* in the biofilm. When the biofilm was allowed to develop for 12 hours prior to the addition of zoocin A to the nutrient media for a further 12 hours, no significant difference was seen in the cfu of any bacterial species. This is likely to be because the biofilm had had time to develop prior to the addition of zoocin A, and cells were therefore more resistant to antimicrobials. Sternberg et al. found that cells in developing biofilms were highly active, whereas cells in the centre of large microcolonies in mature biofilms had a reduced metabolic rate. Many studies have identified that a reduction in metabolism increases resistance to antimicrobials. Also, as the developing biofilm thickens, the presence of EPS may prevent the penetration of antimicrobials into the biofilm. Campanac et al. found that *S. aureus* resistance to quaternary ammonium compounds increased when cells where grown in a biofilm and resistance increased further when the carbon chain length of the antimicrobial compounds was increased. However, susceptibility could be restored to 90% of that of planktonic cells by washing away the biofilm EPS. Mature biofilms are therefore likely to be more resistant to antimicrobials than a developing biofilm, so zoocin A was added for the entire incubation period in all future experiments.

The addition of 40 µg/ml zoocin A for the entire incubation period significantly decreased the cfu of *S. mutans* 10449, when compared to a control biofilm. This is around six times the MIC in broth. However, bacteria growing in biofilms are more resistant to antimicrobials than planktonic bacteria and multispecies biofilms are more resistant than single species biofilms so an increase in resistance to zoocin A was expected. Application of higher zoocin A concentrations (80 and 120 µg/ml) further reduced the cfu when compared to 40 µg/ml zoocin A. However, 120 µg/ml of zoocin A did not give increased inhibition when compared to 80 µg/ml zoocin A, and despite being 20 times the MIC for planktonic cells, *S. mutans* 10449 was still present in the biofilm. Zaura-Arite et al. found that exposure of biofilms to a single chlorhexidine pulse only affected the upper layers of the biofilm, so it
may be that zoocin A is unable to penetrate the biofilm to target cells deeper in the biofilm. These cells are also likely to be less metabolically active and thus more resistant to antimicrobials.\textsuperscript{71,417}

A decrease in the levels of \textit{S. mutans} 10449 was accompanied by an increase in the levels of \textit{S. oralis} 34. This may be due to \textit{S. mutans} 10449 limiting the growth of \textit{S. oralis} 34, possibly by the production of acid. \textit{S. mutans} is particularly acid tolerant and able to continue glycolysis at low pH, which leads to further acid production.\textsuperscript{354,386} \textit{S. oralis} has reduced acid tolerance when compared to \textit{S. mutans}\textsuperscript{426} and may therefore be outcompeted by \textit{S. mutans} growth. The levels of \textit{A. viscosus} T14AV remained unchanged in the presence of zoocin A, suggesting the decrease in cfu in the first 24 hours seen in control biofilms was not due to competition by \textit{S. mutans} 10449, or that the reduced level of \textit{S. mutans} 10449 seen in the presence of zoocin A was not sufficient to allow further \textit{A. viscosus} T14AV growth.

Simmonds et al.\textsuperscript{390} added approximately 8 μg of zoocin A to a biofilm model containing \textit{S. sanguis} 209, \textit{A. viscosus} A69 and \textit{S. mutans} NCTC after four hours of incubation. \textit{S. mutans} NCTC was reduced by approximately two logs directly after incubation with zoocin A for 2 or 30 minutes and this reduction was maintained for up to 20 hours after zoocin A exposure. The levels of \textit{S. sanguis} 209 and \textit{A. viscosus} A69 remained unaffected. The reduction in the cfu of \textit{S. mutans} NCTC caused by a brief exposure to zoocin A in the early stages of biofilm formation seen by Simmonds et al.\textsuperscript{390} was similar to the reduction seen in this study with zoocin A continuously present in the nutrient media. Simmonds et al.\textsuperscript{390} used \textit{S. sanguis} 209 rather than \textit{S. oralis} 34 and did not see an increase in the levels of this streptococcal species when \textit{S. mutans} NCTC was reduced. This suggests \textit{S. sanguis} 209 was not inhibited by the growth of \textit{S. mutans} as \textit{S. oralis} 34 appeared to be. However, the maximum incubation period was 24 hours, which may not have been sufficient to show any alterations in \textit{S. sanguis} 209 growth. Simmonds et al.\textsuperscript{390} found the levels of \textit{A. viscosus} A69 in the control biofilm increased during the 24 hour incubation period, whereas in this study \textit{A. viscosus} T14AV declined in the control biofilm. This is likely to be due to the continuous supply of sucrose in this study producing an acidic biofilm, which subsequently inhibited \textit{A. viscosus} T14AV growth. Simmonds et al.\textsuperscript{390} did not supply sucrose to the biofilm and, although the biofilm pH was not reported, it is likely that the biofilm generated was considerably less acidic. These variations in results emphasise how differences in biofilm models can lead to different outcomes and show that a simple biofilm model cannot model all of the complex interactions that occur when many different species are present.
Lauricidin was applied to the biofilm at various concentrations to identify a concentration suitable for use in combination with zoocin A. As for planktonic cells, lauricidin was able to target and significantly reduced the cfu of all three species when applied at 15 and 30 µg/ml. Unlike zoocin A, concentrations of lauricidin similar to the MIC against planktonic cells were sufficient to significantly reduce the bacterial levels, suggesting biofilm formation does not offer the same level of protection from lauricidin. This may be due to more effective penetration of the biofilm by lauricidin compared to zoocin A. Lauricidin (molecular weight 274.45) is considerably smaller than zoocin A (molecular weight 27,877) and smaller molecules are often more effective at biofilm penetration. However, other factors such as chemical interaction with the EPS also impact upon antimicrobial penetration of the biofilm. As for zoocin A, a population of cells survived the antimicrobial treatment despite increasing antimicrobial concentrations. This suggests there is a limitation on the effectiveness of antimicrobials on the biofilm. This may be due to limitations on the penetration of the antimicrobials into the biofilm or a subpopulation of cells which are resistant, probably due to their reduced growth rate within the biofilm.

Lauricidin concentrations of 10 µg/ml significantly reduced the cfu of S. mutans 10449 in the biofilm model, but did not affect the levels of S. oralis 34 and A. viscosus T14AV. This result differs from their relative sensitivities to lauricidin in broth culture, where S. mutans 10449 was slightly more resistant to lauricidin. This may be due to interactions between S. mutans and the other bacteria in the biofilm. Several studies have found that non-cariogenic bacteria in a mixed species biofilm can impact upon the growth and virulence of S. mutans. Studies have found that acid production increased when S. mutans was grown in a biofilm and Kabara and Vrable found S. mutans was more sensitive to monolaurin as the pH decreased. S. mutans may therefore be more sensitive to lauricidin when grown in a biofilm.

Unlike the response seen with zoocin A, the bacterial count of S. oralis 34 did not increase with decreasing levels of S. mutans 10449, indicating the inhibitory effects of lauricidin on S. oralis 34 are sufficient to overcome any increase growth due to reduced competition from S. mutans 10449. A lauricidin concentration of 10 µg/ml was selected for further experiments, as this concentration reduced S. mutans but did not affect S. oralis 34 and A. viscosus T14AV. It also gave a similar reduction in S. mutans 10449 as 40 µg/ml zoocin A, which would allow any increase in inhibition caused by both antimicrobials in combination to be seen.
6.3.4 Zoocin A and lauricidin in combination

When zoocin A and lauricidin were added to the biofilm model in combination S. mutans 10449 was reduced by nearly four logs compared to the control biofilm after 24 hours. This was significantly more than the reduction seen with zoocin A or lauricidin alone (approximately 1.3 and 1.9 logs respectively). Numerous studies have investigated the effects of antimicrobial combinations on S. mutans. Koo et al. grew S. mutans biofilms for 24 hours and then pulsed (twice daily over four days) the biofilms with combinations of fluoride (250 ppm), tt-Farnesol (5 mM) and apigenin (1 mM). Changes in biofilm growth were then examined, including bacterial cfu, total biomass and total protein content. Treating the biofilm with fluoride, tt-Farnesol and apigenin in combination significantly reduced the cfu of S. mutans when compared to a control biofilm. However, this reduction was only 0.3 logs and was not significantly different than the effect of the antimicrobials tested when applied individually or in pairs. This reduction in S. mutans cfu was considerably less than the reduction achieved in this study with lauricidin and zoocin A, but there were several differences between the studies. The biofilms grown by Koo et al. were single species biofilms, which would be expected to be more susceptible to antimicrobials than multi-species biofilms. However, the biofilms were allowed to develop for 24 hours prior to the addition of the antimicrobial and established biofilms are more resistant to antimicrobial effects. An earlier study by Koo et al. with similar methodology compared the effects of tt-Farnesol and apigenin with chlorhexidine at the same molar concentration (1.3 mM) and found chlorhexidine was significantly more effective than tt-Farnesol and apigenin in combination. Chlorhexidine reduced the cfu of S. mutans by approximately five logs, while tt-Farnesol and apigenin reduced the cfu of S. mutans by around one log. In this study chlorhexidine at 11.1 µM also reduced the cfu of S. mutans by approximately five logs after 24 hours of incubation, while the combination of zoocin A and lauricidin reduced the cfu of S. mutans around four logs. Although this suggests zoocin A and lauricidin may be a more effective antimicrobial combination, differences in methodology and the concentration of chlorhexidine used makes direct comparisons inconclusive. Many other studies have investigated antimicrobial combinations and their anti-cariogenic potential, such as chlorhexidine and fluoride or xylitol, and triclosan and fluoride or zinc citrate. However, large differences in methodology, in particular how biofilm or S. mutans inhibition was quantified, makes meaningful comparison impossible.
For *S. oralis* 34 and *A. viscosus* T14AV the effect of zoocin A and lauricidin in combination was similar to that of lauricidin alone after 24 hours of incubation. The combination of zoocin A, a mutans-specific inhibitory agent, with lauricidin, a relatively non-specific agent, resulted in significantly increased inhibition of *S. mutans* 10449 without increased inhibition of the non-target species (Table 6.1).

### 6.3.5 The pH of biofilms exposed to lauricidin and zoocin A

A crucial component in the formation of dental caries is the production of acid which causes demineralisation of the plaque.268,354 The lowering of the pH, which arises from acid production, not only results in enamel demineralisation, but also selects for acid tolerant bacteria.448 These acid tolerant bacteria maintain glycolytic processes at low pH, which in turn provides energy to maintain the internal pH of the cell, again allowing glycolytic processes to continue at low pH.42,386 Therefore, an outcome of an anti-caries strategy must be a reduction in acid formation, to prevent the initiation of this cycle. Preventing the reduction in pH could indicate the biofilm ecology has been sufficiently altered to prevent a cariogenic state. The pH of the biofilm cells was therefore recorded. After 72 hours the pH of the control biofilm reached 4.3, while the pH of biofilms exposed to zoocin A and lauricidin in combination was consistently maintained above 7.0. Exposure to the antimicrobials individually maintained the pH above 6.0. While the pH at which teeth begin to demineralise varies, it is in the range of 5.3 to 5.7,98 suggesting the maintenance of the pH seen in the biofilm model when it is exposed to these antimicrobials would be sufficient to prevent tooth demineralisation, if results translated to the oral cavity.

Duarte et al.114 tested the ability of cranberry polyphenols to prevent a pH drop by *S. mutans* cells after glucose challenge and found 30 minutes after glucose challenge the pH of the control was reduced to 4.91, while *S. mutans* in the presence of a combination of polyphenols had a pH of 5.12. Petersen et al.340 investigated the antimicrobial effects of a combination of sodium fluoride (58 mM) and sodium lauryl sulphate (SLS) (0.5 mM). *S. mutans* biofilms were grown in microtitre plates for 20 hours then exposed to the test antimicrobials for five minutes before analysis. Although bacterial cfu was not measured, the combination reduced lactic acid production in the biofilm around 10-fold compared to a control treated with PBS. Lactic acid production was also significantly reduced when compared to the effect of either antimicrobial alone.
Table 6.1: Summary of changes in the biofilm on addition of antimicrobials

| Bacterial strain | Log change\textsuperscript{a} in cfu after 24 hours incubation in the presence of: |  
| --- | --- | --- |
|  | Zoocin A (40 µg/ml) | Lauricidin (10 µg/ml) | Zoocin A (40 µg/ml) and lauricidin (10 µg/ml) |
| S. mutans | -1.3 | -1.9 | -3.9 |
| S. oralis | + 2.5 | NC\textsuperscript{b} | NC |
| A. viscosus | NC | NC | NC |

\textsuperscript{a} Data from Figure 5.4

\textsuperscript{b} No significant difference in cfu recorded as no change (NC)
The difference in pH between treated and untreated groups was greater in this study; however, Petersen et al. measured lactic acid production rather than overall pH, and substantial differences in the methods between this study and that of Duarte et al. make direct comparison difficult. These studies were also both done on single species biofilms and Kara et al. found a single species biofilm of *S. mutans* produced more lactic acid than a dual species biofilm of *S. mutans* and *Veillonella parvula*, suggesting bacterial interactions in a biofilm may reduce acid production.

While *S. mutans* can maintain glycolytic processes at pH 4, and survive at pH levels as low as 2.5, most bacteria cannot grow at pH levels below five. In the control biofilm the pH dropped below five between 24 and 48 hours, while in the biofilms exposed to antimicrobial treatments the pH remains above five. The lowering of the pH in the control biofilm is likely to be the cause of the reduction of *S. oralis* in the control biofilms. This is supported by the fact that *S. oralis* levels are elevated compared to the control biofilm when biofilms are exposed to antimicrobials, especially after 48 and 72 hours, where the difference in pH between the biofilms is more distinct. A similar effect can be seen with *A. viscosus* T14AV.

### 6.3.6 Biofilm formation

Changes in the visual appearance of the biofilms suggested that antimicrobial treatment greatly reduced biofilm formation. To quantify this reduction a fluorescent lectin was used. Fluorescently labelled lectins bind to carbohydrates with characteristic specificity. Fluorochrome conjugated lectins have been used to visualise the EPS of biofilms by epifluorescence microscopy and to quantify the N-acyl glucosamine component of *S. epidermidis* biofilms. The wheat germ agglutinin lectin is isolated from *Triticum vulgaris*. It binds N-acetyleneuraminic acid in the peptidoglycan layer of gram-positive bacteria and to polysaccharide adhesin (poly-N-acetylglucosamine), which is involved in biofilm formation by both gram-positive and gram-negative bacteria. Fluorescent production was increased when *S. mutans* cells were grown in the presence of sucrose, which results in biofilm formation by the cells, indicating the fluorescent lectin can be used to investigate biofilm formation. This is supported by the findings of Burton et al. who compared labelling a biofilm with FITC-WGA to the use of crystal violet for biofilm staining and found a strong correlation between the two methods. Labelling with the fluorescent lectin was also found to be more sensitive and specific than crystal violet staining in determining *E. coli* and *S. epidermidis* biofilm inhibition by various antimicrobial agents.
Using the fluorescent lectin to label the control biofilm and those exposed to zoocin A, lauricidin, or both in combination, confirmed the reduction in biofilm formation seen when examining the visual appearance of the biofilms, although no difference between the different antimicrobial treatments could be seen. Wei et al.\textsuperscript{467} used crystal violet staining to examine biofilm inhibition. \textit{S. mutans} biofilms were treated with various low molecular weight salivary mucins (MUC7 peptides) by growing \textit{S. mutans} in BHI containing the antimicrobial peptide for 24 hours in a microtitre plate. Planktonic cells were then removed and the biofilms were washed. Biofilms were examined by analysing biofilm inhibition, using the absorbance to calculate the percentage of inhibition. At concentrations above 12.5 µM most MUC7 peptides reduced biofilm formation by over 90% compared to the untreated biofilms. In this study the biofilms incubated in the presence of zoocin A (1.33 µM) and lauricidin (36.5 µM) for 24 hours were inhibited by around 70% and after 72 hours this reduction was increased to over 85%. When Wei et al.\textsuperscript{467} incubated the biofilms for 24 hours then added the antimicrobial peptides to the nutrient media for a further 24 hours, biofilm inhibition was considerably reduced. Some peptides reduced biofilm formation by a maximum of 40% when added at 12.5 µM, although for several peptides no reduction in biofilm formation was seen. This is similar to results in this study where the addition of zoocin A to the biofilm for 12 hours, after 12 hours of incubation without antimicrobial addition, did not result in a decrease in the cfu of \textit{S. mutans}, probably as established biofilms are more resistant to antimicrobials.\textsuperscript{71,417}

Other studies have quantified the amount of EPS or insoluble glucans present after biofilm treatment. Duarte et al.\textsuperscript{114} treated 24-hour-old biofilms by submersing them in combinations of cranberry polyphenols (125 mg/ml flavonols, 200 mg/ml anthocyanins and 500 mg/ml proanthocyanidins) for one minute before rinsing and returning them to culture media. Treatment occurred twice daily for four days and significantly reduced the accumulation of total biomass by around 20% and insoluble glucans by approximately 35%. This was correlated with an inhibition of GTF B and C. Petersen et al.\textsuperscript{340} isolated bacteria from saliva samples and measured the production of polysaccharides by these cells when grown in a biofilm. Production was significantly reduced by around 20% when patients were using a mouthwash containing sodium fluoride (58 mM) and SLS (52 mM). Plaque from these individuals also had significantly reduced cfu per mg of sample. While these reductions in biofilm formation are smaller than those seen in this study, these studies measured the changes in specific polysaccharides, rather than overall biofilm formation, and this makes comparisons inconclusive.
To further examine changes in biofilm formation after exposure to antimicrobials scanning electron microscopy was used. This again confirmed biofilm formation was reduced by exposure to antimicrobials. Wei et al.\textsuperscript{467} also examined biofilms by scanning electron microscopy. Biofilms were grown on hydroxyapatite discs and in a Calgary biofilm device, and were either untreated or exposed to the various MUC7 peptides. Untreated biofilms grown on hydroxyapatite disc displayed continuous microbial growth, with large gaps and channels in the biofilm. The continuous nature of the biofilm was similar to the control biofilms in this study, but in this study channels in biofilm were not seen. The untreated biofilms grown using a Calgary biofilm device by Wei et al.\textsuperscript{467} appeared as discrete biofilm colonies that did not cover the substrate, but the growth in these biofilms was thick and continuous, similar to the control biofilms in this study. Treatment with 50 µM of a MUC7 peptide (four times the MIC) reduced the biofilms to small clumps of cells and residual EPS on both substrates,\textsuperscript{467} similar to the effect of zoocin A and lauricidin seen in this study.

Biofilms exposed to zoocin A appeared very fragmented and flaky at both high and low magnification. The presence of zoocin A appeared to prevent the formation of dense, continuous biofilms. Since the development of dense biofilms reduces the effectiveness of antimicrobials,\textsuperscript{71,417} treatment with zoocin A may allow other antimicrobials to be more effective. Biofilms exposed to lauricidin were patchy, but still contained areas of dense microbial growth, which may remain resistant to antimicrobial treatment. If the presence of zoocin A prevents the formation of a dense biofilm structure this may allow lauricidin more access to the biofilm cells, improving the antimicrobial effect and lead to the increased effect seen on biofilms treated with both zoocin A and lauricidin.

\textbf{6.3.7 Addition of chlorhexidine to the biofilm model}

The large variation in biofilm models makes it difficult to compare results between studies on different antimicrobials. It is therefore important to include a control or standard to compare with the effect of a new antimicrobial. Chlorhexidine is used in some commercially available dental products and has been extensively investigated as an antimicrobial for the oral cavity.\textsuperscript{22,438} Chlorhexidine is therefore often used as a positive control or to benchmark the effectiveness of new antimicrobials.\textsuperscript{14,25} In this study chlorhexidine digluconate was added to the biofilm model at 10 and 50 µg/ml (0.001 and 0.005%). Chlorhexidine was used at 10 µg/ml, which equates to 11.1 µM of chlorhexidine. This was within the range of the molar concentrations of zoocin A and lauricidin, which were 1.33 µM and 36.5 µM respectively.
Chlorhexidine was also used at 50 µg/ml (55.7 µM) as this is the highest concentration that was soluble in BHI.

After 24 hours, 10 µg/ml of chlorhexidine reduced *S. mutans* 10449 by over five logs compared to the untreated biofilm. This reduction was less pronounced after 48 and 72 hours, but remained over 3.5 logs. The effects of 50 µg/ml chlorhexidine were greater, but followed a similar pattern. While several other studies have investigated the effects of chlorhexidine on a biofilm model, higher concentrations are usually required to reduce the bacterial count of *S. mutans* in a biofilm. Deng et al.\(^\text{104}\) found at least 0.0075% was required to reduce *S. mutans* levels by one log when grown in a biofilm. Al-Ahmed et al.\(^\text{9}\) grew *S. mutans* in a biofilm reactor and found 0.2% chlorhexidine resulted in close to a three log reduction in cfu when compared to an untreated biofilm. However, in the studies by Deng et al.\(^\text{104}\) and Al-Ahmed et al.\(^\text{9}\) chlorhexidine was not added until after 24 and 96 hours of incubation respectively, whereas in this study antimicrobials were added to the nutrient media for the entire incubation period, which may account for the large reduction seen in cfu with relatively low chlorhexidine concentrations. The maximum inhibition of the bacterial count was seen after 24 hours of incubation, while after 48 and 72 hours of incubation the levels of *S. mutans* 10449 and *S. oralis* 34 partially recovered. This may be due to growth of cells which are able to tolerate, or are resistant to, these low chlorhexidine concentrations. It has been shown that in other bacterial strains repeated exposure to subinhibitory concentrations of chlorhexidine leads to resistant strain formation.\(^\text{430,434}\)

Changes in biofilm formation were measured using FITC-WGA and the reduction was similar to that seen with zoocin A, lauricidin and both in combination. After 24 hours of incubation the amount of fluorescence recorded from biofilms treated with chlorhexidine at 10 or 50 µg/ml was significantly lower than that of untreated biofilms, or those treated with zoocin A, lauricidin, or both in combination. This suggests that after 24 hours chlorhexidine treatment gives a greater reduction in biofilm formation. However, this did not occur after 48 and 72 hours of incubation, where differences between the different biofilm treatments could not be seen. Altman et al.\(^\text{14}\) compared the effect of 5 to 500 µg/ml chlorhexidine to the action of three antimicrobial peptides against *S. mutans* grown in a biofilm. The antimicrobial treated biofilms were examined using confocal scanning laser microscopy and measured the percentage of viable cells in the biofilm. The appearance of the biofilms did not alter with the addition of the antimicrobials, whereas in this study biofilm formation was reduced. However, Altman et al.\(^\text{14}\) found the percentage of viable cells was substantially reduced.
Chlorhexidine and the K₄-S₄(1-15)a peptide added individually at 500 μg/ml reduced the percentage of viable cells to under 10%, compared to 99% in the untreated biofilm. Biofilms treated with 5 or 50 μg/ml chlorhexidine or K₄-S₄(1-15)a had over 80 and 60% of viable cells respectively. These biofilms were grown overnight on glass cover slips covered in BHI supplemented with sucrose and then antimicrobials were added for one hour prior to analysis. The results of Altman et al.¹⁴ suggest the addition of chlorhexidine to established biofilms is able to reduce biofilm viability, while the presence of chlorhexidine throughout the incubation period prevents biofilm development.

Overall the addition to chlorhexidine to the biofilm reduced the levels of S. mutans 10449, S. oralis 34 and A. viscosus T14AV, especially after 24 hours, and prevented the pH reduction seen in the control biofilm. However, no significant difference in pH can be seen between treatment with chlorhexidine or zoocin A, lauricidin, and zoocin A and lauricidin in combination. Although the application of chlorhexidine resulted in a greater reduction in the cfu of S. mutans 10449 than zoocin A and lauricidin, few differences in the degree of biofilm inhibition or pH levels can be seen. This suggests that the inhibition of S. mutans achieved by the addition of zoocin A and lauricidin may result in a similar overall effect as chlorhexidine. However, the application of zoocin A and lauricidin was more targeted, only reducing the levels of S. mutans 10449, while levels of S. oralis 34 were increased and A. viscosus T14AV unchanged. This targeted approach may be advantageous as non-cariogenic bacteria in the oral cavity are beneficial²⁹⁵,²⁹³ and targeted antimicrobials may be less disruptive for the oral microflora. This can leave a non-cariogenic biofilm intact, which may reduces recolonisation by S. mutans.²⁵⁶

6.3.8 Addition of mouthwash to the biofilm model

Many different mouthwashes are commercially available and marketed as antimicrobial products. Diluted mouthwash was added to the biofilm model to compare the effects of zoocin A and lauricidin to commercially available products. Listerine FreshBurst antiseptic mouthwash contains the essential oils thymol (0.64 mg/ml) and cineole (eucalyptol) (0.92 mg/ml) as the active ingredients. It also contains 22% ethanol and 1.5 mg/ml benzoic acid. Savacol antiseptic mouthwash contains 2 mg/ml chlorhexidine gluconate as the active ingredient and 11.5% ethanol. The mouthwashes were added to the biofilm nutrient media at a concentration of 1% as this brought the concentrations of the active ingredients into the μM range, similar to the concentrations of zoocin A and lauricidin used (1.33 μM and 36.5 μM
respectively). When Listerine FreshBurst antiseptic mouthwash was added to the biofilm the concentrations of thymol and cineole were 42.6 µM and 5.97 µM respectively, while the addition of 1% Savacol antiseptic mouthwash resulted in 22.3 µM of chlorhexidine gluconate.

Neither the presence of 1% Listerine FreshBurst antiseptic mouthwash nor the matched ethanol control reduced the bacterial count of the biofilms or prevented the decrease in pH seen in the control biofilms. While biofilm formation did appear to be reduced by the presence of 1% Listerine FreshBurst antiseptic mouthwash, this reduction was not statistically significant. The lack of effect of Listerine FreshBurst antiseptic mouthwash on the biofilm model may be due to insufficient concentrations of the active ingredients being present. Shapiro et al.\textsuperscript{383} compared a variety of commercially available mouthwashes and found Listerine was the most effective herbal or phenolic based product. Five and six species biofilms were grown in 24-well cell culture plates and pulsed with undiluted mouthwash six times during 64.5 hours of incubation. Listerine reduced the total bacterial cfu by approximately 2.5 logs.

The chlorhexidine containing mouthwash was more effective. The addition of 1% Savacol antiseptic mouthwash to the biofilm model resulted in a 3.0 log decrease in the level of \textit{S. mutans} 10449, slightly less than the reduction seen with zoocin A and lauricidin used in combination, which was 3.9 logs. The final concentration of chlorhexidine when 1% Savacol antiseptic mouthwash was added to the media (22.3 µM) was within the range of the two concentrations of chlorhexidine digluconate used (11.1 and 55.7 µM). However, after 24 hours of incubation a greater inhibition of \textit{S. mutans} was seen with 11.1 µM chlorhexidine digluconate added directly to the nutrient media than with 1% Savacol antiseptic mouthwash. Like chlorhexidine digluconate, the presence of 1% Savacol antiseptic mouthwash also reduced the cfu of \textit{S. oralis} 34. Shapiro et al.\textsuperscript{383} found chlorhexidine containing mouthwashes were the most effective, reducing the bacterial count of a multispecies biofilm model by at least four logs. Chlorhexidine is effective against a range of bacterial species and therefore acts against numerous species in a biofilm model, whereas the combination of zoocin A and lauricidin specifically targets \textit{S. mutans}.

While Listerine FreshBurst antiseptic mouthwash did not significantly alter biofilm formation or pH the addition of Savacol antiseptic mouthwash gave results similar to both chlorhexidine digluconate and zoocin A and lauricidin in combination. This again suggests that the
application of zoocin A and lauricidin may sufficiently alter the biofilm, by reducing biofilm formation and acid production, to have a similar overall effect as chlorhexidine.

6.3.9 The development of resistance after exposure to zoocin A and lauricidin

The development of resistance to antimicrobial treatments is a concern as it can lead to difficulties in treatment and increased treatment costs. Antimicrobials found in oral care products such as toothpastes and mouthwashes are used for extended periods of time and do not eliminate the resident microbial population. This would be expected to create an environment that selects for resistance development, and bacteria resistant to common oral antimicrobials such as fluoride and xylitol have been found in the oral cavity. It was therefore important to investigate the development of resistance to zoocin A and lauricidin in the biofilm model. A single colony of *S. mutans* 10449 (designated colony A) obtained from a biofilm exposed to 10 µg/ml lauricidin, demonstrated increased resistance to lauricidin. No increase in resistance was seen for *S. mutans* 10449 obtained from biofilms exposed to no antimicrobial, zoocin A, or zoocin A and lauricidin in combination, or for *S. oralis* 34 and *A. viscosus* T14AV from any biofilm. These results indicate the development of resistance to zoocin A or lauricidin did not occur with high frequency.

The resistance of colony A was further investigated to determine the extent of the resistance and whether it was maintained after lauricidin exposure was halted. Colony A was subcultured from the 180 µg/ml lauricidin agar into nonselective and lauricidin-containing broth. The colony A cultures from both lauricidin-containing and nonselective broths were used to calculate the MIC of lauricidin and zoocin A individually. This was compared to the MIC for wild type *S. mutans* 10449. When colony A was maintained in lauricidin containing broth the MIC of lauricidin was increased compared to both the wild-type *S. mutans* 10449 and colony A maintained in nonselective broth. However, the increase in the MIC of lauricidin was from 15.63 to 31.25 µg/ml, which suggests the increase in resistance to lauricidin was not large. These results suggest the increase in resistance to lauricidin was maintained only with continued exposure to lauricidin and on removal of lauricidin the cell reverted to the wild type sensitivity. Reversion to antimicrobial sensitivity was also seen by Streckfuss et al., where fluoride resistant strains of *S. mutans* were isolated from patients receiving daily 1% NaF gels. All these strains reverted to fluoride sensitivity after subculturing in the laboratory.
Similar results were seen when the MIC of zoocin A for colony A was determined. The MIC of wild-type *S. mutans* 10449 and colony cultured without lauricidin was the same (6.25 µg/ml), while the MIC of colony A cultured with lauricidin was increased to 12.5 µg/ml. This again indicates that the increase in resistance is lost when selective conditions are removed and suggests that the mechanism of increased resistance to lauricidin also increases resistance to zoocin A. Tattawasart et al.\(^{430}\) found that resistance to chlorhexidine by *Pseudomonas stutzeri* also lead to increased resistance to other antimicrobials such as triclosan and EDTA and these changes were attributed to changes in the cell envelope.

The lag phase of wild-type *S. mutans* 10449 appeared to be longer than the lag phase of colony A, as the wild-type *S. mutans* 10449 grown in BHI took one to four hours longer than colony A to reach an OD\(_{595}\) of 0.4. This is an unusual result, as generally an increase in resistance would be expected to result in a decreased growth rate.\(^{97,125}\) As this occurs when colony A is grown in BHI alone and BHI with a range of zoocin A and lauricidin concentrations, it cannot be linked to the presence of either antimicrobial.

Colony A grown in an agar stab was able to maintain increased lauricidin resistance, regardless of the presence of lauricidin, whereas it did not grow when struck on lauricidin-containing agar. Colony A also did not maintain increased resistance when struck on nonselective agar or when used to inoculate BHI broth and then returned to lauricidin containing agar as a stab culture. This supports the suggestion that the increase in resistance to lauricidin was maintained only with continued exposure to lauricidin and that on removal of lauricidin the cell reverted to the wild-type sensitivity. It also suggests that growth conditions are also a factor in maintaining the increased antibiotic resistance as growth of colony A in an agar stab appears to maintain the increased resistance, while growth on the agar surface does not. Stab and streak cultures were incubated under the same conditions; however, growth conditions at the bottom of the stab would be anaerobic, while growth on the agar surface would be exposed to limited oxygen. These differences in growth conditions may affect the maintenance of increased resistance to lauricidin.

Overall these results suggest resistance is unlikely to occur with high frequency and is unlikely to be maintained after antimicrobial exposure is halted. However, it is possible that this investigation was unable to detect resistance development as the colonies tested represent a very small sample of the total biofilm. It is also possible the incubation period was insufficient to allow any advantageous resistance to become dominant in the plaque. As the
increased resistance to lauricidin seen in colony A also resulted in increased resistance in zoocin A resistance may be due to transient changes in the cell envelope. Resistance is less likely to occur when antimicrobials are used in combination and no increase in resistance was seen when zoocin A and lauricidin were used in combination.

6.4 Conclusions and further studies

6.4.1 Production of zoocin A

Zoocin A was produced in a 10 L culture without a reduction in the overall protein yield. This produced 1650 mg of zoocin A per 10 L of culture. However, when using the biofilm model, 24 hours of incubation of three biofilms with 40 µg/ml zoocin A in the nutrient media requires 160 mg zoocin A and therefore many 10 L batches are required to carry out multiple experiments using zoocin A. To further investigate the use of zoocin A as an antimicrobial, especially at higher concentrations, larger amounts zoocin A would be required. Since codon usage does not appear to be a limiting factor in the production of zoocin A, high cell-density culture could be investigated. As zoocin A is accumulated intracellularly in \textit{E. coli}, protein productivity is proportional to the final cell density. To achieve a high cell-density culture fed batch cultures are used. High concentrations of some nutrients and culture by-products, such as acetate, can inhibit cell growth when present at high levels and feeding techniques are therefore used to keep nutrients and acetate at acceptable levels. Feeding strategies may be constant, exponential or coupled to culture parameters such as dissolved oxygen, pH, carbon dioxide evolution rate, cell concentration (indirect feedback) or substrate concentration (direct feedback). In this way, higher cell densities of \textit{E. coli} could be achieved which would result in higher concentrations of protein from the same culture volume.

6.4.2 Future development of the biofilm model and the use of zoocin A and lauricidin as antimicrobials

The biofilm model developed in this study was simple, reproducible and stable for at least 72 hours. There are many different biofilm models used to test the effectiveness of various antimicrobial agents and this makes comparisons between studies difficult. Although the use of chlorhexidine as a positive control allows the benchmarking of the activity of an antimicrobial against a standard, differences in the biofilm species, incubation time,
antimicrobial treatment protocol and methods used to quantify biofilm inhibition of zoocin A reduction make direct comparisons between novel antimicrobials challenging. This biofilm model could be further characterised by investigations into the early formation of the biofilm and the interactions between the three species present in the biofilm. The effect of inoculation levels and the order of inoculation of the species could also be investigated.

It was found that zoocin A in combination with lauricidin had an increased effect on the bacterial count of *S. mutans* 10449 compared to either antimicrobial individually. However, from these results it was not possible to determine the type of interaction between zoocin A and lauricidin. Since zoocin A acts upon the cell wall and lauricidin acts upon the cell membrane, it might be expected that the antimicrobials act synergistically. To determine the type of interaction between zoocin A and lauricidin a checkerboard analysis could be used. The fractional inhibitory concentration (FIC) for each antimicrobial is calculated using the MIC of each antimicrobial, both individually and in combination. The FIC equals the MIC of an antimicrobial alone divided by the MIC of the antimicrobial in combination with another. The FIC values are then used to calculate the FIC index, which is equal to the FIC of the first antimicrobial plus the FIC of the second. An FIC index of less than 0.5 is defined as a synergistic interaction, an FIC of 0.5 to 4 is defined as indifference and an FIC of greater than 4 is an antagonistic relationship. This method was used by Filoche et al. to determine the relationship between chlorhexidine and essential oils when used in combination upon *S. mutans*. Further studies could also be carried out on the development of resistance to the antimicrobials. This would involve analysing the nature of the decreased susceptibility and investigating whether continued exposure, or changes in antimicrobial concentrations or treatments, increases the incidence of resistance formation.

The antimicrobial agents were added to the nutrient media for the entire incubation period, as this was a simple and consistent way of introducing them to the biofilm model. The addition of zoocin A to the biofilm model for 12 hours, after the biofilm model had been pre-incubated for 12 hours, did not reduce the levels of *S. mutans* 10449, suggesting it was unable to affect biofilms that were already established. However, oral care products are usually used during, or directly after, toothbrushing and antimicrobial agents are therefore acting during the initial stages of biofilm development, or when the microbial load is reduced. It would therefore be interesting to establish whether antimicrobial agents present at the beginning of incubation and then removed from the nutrient media had a long-term effect on bacterial cfu and biofilm pH. This was seen by Simmonds et al. when zoocin A was added to biofilms after four
hours of incubation. The bacterial count of *S. mutans* was still reduced after a further 20 hours of incubation. The effects of brief pulses of an antimicrobial agent throughout biofilm development could be investigated. Rukayadi and Hwang\(^{367}\) investigated the effect of coating the biofilm substrate in xanthorrhizol prior to the inoculation of the biofilm bacteria, and Deng et al.\(^{106}\) used twice daily pulses of fluoride and fluoride with chlorhexidine to investigate their effects on dentin remineralisation. Different combinations of antimicrobial concentrations could also be trialled. In this study concentrations of lauricidin and zoocin A that gave similar reductions in *S. mutans* 10449 were used and concentrations of lauricidin were kept low enough to not reduce *S. oralis* 34 and *A. viscosus* T14AV. This was to keep the antimicrobial effect targeted to *S. mutans*. However, as the presence of zoocin A and subsequent reduction in *S. mutans* was found to lead to an increase in *S. oralis* 34 levels, higher concentrations of lauricidin in combination with zoocin A may not result in a reduction of the non-cariogenic bacteria in this model.

Although many different antimicrobial treatment regimens could be used, this biofilm model is simple and is limited in its ability to mimic the oral cavity by its simple broth nutrient source and limited number of species. In-depth investigations of the effects of zoocin A and lauricidin on *S. mutans* could be carried out in a more complex model. However, ultimately the effectiveness of these antimicrobials would need to be investigated using human trials. This would establish whether the effects of the antimicrobials seen in a biofilm model are sufficient to reduce dental caries. Human trials would require zoocin A to be established as non-toxic for eukaryotic cells and approved for use in humans, while lauricidin is already available commercially as an oral antimicrobial. Many other factors would also need to be investigated prior to human trials. The optimal dosage of each antimicrobial would need to be established, along with the optimal time during biofilm formation for the antimicrobials to be introduced. The persistence and stability of zoocin A and lauricidin in the oral cavity would also need to be investigated. A preliminary study by Simmonds et al.\(^{390}\) found that zoocin A activity was not reduced by salivary components and that saliva appeared to have a stabilising effect on the zoocin A molecule. A delivery system to introduce the antimicrobials to the oral cavity would also be required. This could be achieved using chewing gum. Medicated chewing gums can be used as a drug delivery system as the active ingredients are released during chewing and then the gum can be discarded.\(^{287}\) In comparison with several other delivery systems, chewing gum is kept in the mouth longer and can result in prolonged drug release and contact with the teeth.\(^{317,334,490}\) Gum offers the added advantage of increasing salivary secretion when chewed\(^{184}\) and chewing gums are a
commonly used commodity which are readily accepted by patients, improving compliance.\textsuperscript{287,490} Chewing gums can be produced by direct compression. These chewing gums can contain higher levels of active ingredients than traditional gums and they have a lower production temperature, which can help avoid inactivation of bioactive molecules. They also have reduced moisture content, which can improve the stability of these molecules.\textsuperscript{287} These properties would allow zoocin A to retain its activity in the chewing gum delivery system and both zoocin A and lauricidin could be released into the oral cavity over a period of time, increasing antimicrobial exposure time. Studies in the oral cavity could also examine the effects of zoocin A and lauricidin treatment on the oral pH after sucrose challenge and on the development of resistance after long term exposure to the antimicrobials.

6.4.3 Conclusions

The oral microflora contains many different bacterial species\textsuperscript{2} and forms an intricate and structured biofilm.\textsuperscript{231,244} \textit{S. mutans} has been shown to be a prime etiological agent of dental caries\textsuperscript{294,445} that is particularly acidogenic and aciduric,\textsuperscript{354} and this acid production results in a low pH in the oral cavity and demineralisation of the tooth enamel.\textsuperscript{310,449} Prevention of dental caries aims to prevent dental plaque build up or to reduce the levels of mutans streptococci in the plaque.\textsuperscript{28,415} Many different antimicrobials have been investigated as anti-caries agents. This study investigated the effects of zoocin A and lauricidin upon an oral biofilm model. Zoocin A is an endopeptidase which cleaves peptidoglycan,\textsuperscript{138} rupturing the cell wall,\textsuperscript{391} and is active against \textit{S. mutans}.\textsuperscript{188,390} Lauricidin is a distilled monoglyceride, consisting of 90\% monolaurin and is active against a range of gram-positive bacteria, including streptococci.\textsuperscript{202,203}

This study has achieved its aims of: producing zoocin A in sufficient concentrations to use as an antimicrobial in a biofilm model and optimising the protein expression in a recombinant \textit{E. coli} system; developing a triple-species biofilm model suitable for investigating the effects of antimicrobials on an oral biofilm; and using the biofilm model to study the effects of zoocin A and lauricidin, both individually and in combination, upon a biofilm and comparing these results with the effects of known anti-plaque agents. It also confirmed the hypothesis that zoocin A can be used to change the ecology of a plaque biofilm model.
The triple-species biofilm model was reproducible and dynamic. It was able to distinguish between a cariogenic and non-cariogenic state, as the levels of *S. mutans* in the biofilm could be enumerated, and the pH and biofilm formation measured. The levels of *S. mutans* in the biofilm has been correlated to caries risk. The combination of zoocin A and lauricidin was able to target *S. mutans* in the biofilm model, while not reducing either *S. oralis* or *A. viscosus* and was significantly more effective against *S. mutans* than either antimicrobial alone. The combination prevented the development of a cariogenic state as evidenced by: the reduced levels of *S. mutans*, the reduced biofilm formation, and the prevention of the pH decrease seen in the control biofilms. Exposure to zoocin A and lauricidin in combination for 72 hours did not select for the development of resistance. When the effects of this antimicrobial combination were compared to similar concentrations of chlorhexidine, chlorhexidine was found to produce a greater reduction in *S. mutans* in the first 24 hours of incubation. Chlorhexidine also reduced the cfu of the other bacterial species, while the effects on pH and biofilm formation were similar to those seen with zoocin A and lauricidin. Similar results were seen with a chlorhexidine containing mouthwash, whereas a mouthwash containing essential oils as the active ingredients did not reduce the bacterial levels of the biofilm model. Zoocin A and lauricidin were therefore able to selectively reduce *S. mutans* in the biofilm model, while chlorhexidine had a broader antimicrobial effect.

Targeted antimicrobials are considered beneficial as they may be less disruptive for the beneficial oral microflora and combinations of agents can both increase the antimicrobial effect and reduce the development of resistance. However, extensive further study is required to establish whether these agents could be used as effective antimicrobials for the oral cavity.
7. References


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